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## STUDIES IN HISTOCHEMISTRY

### VII. THE CONCENTRATION OF VITAMIN C IN THE THYMUS IN RELATION TO ITS HISTOLOGICAL CHANGES AT DIFFERENT STAGES OF DEVELOPMENT AND REGRESSION

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San Francisco)

#### PLATE 1

(Received for publication, January 30, 1936)

In some of the previous papers of this series, the histological distribution of vitamin C in certain endocrine organs was investigated. Studies were made of the adrenal (1), hypophysis (2), and corpus luteum (3). The present investigation extends this work to include the thymus gland.

Von Euler and Klusmann (4) reported the vitamin C concentration of the thymus but expressed their results in cc. of 2,6-dichlorophenol indophenol solution required in the titration of the ascorbic acid in 1 gm. of tissue, without giving the ascorbic acid equivalent of the dye solution. They record values of 1.4 to 2.6 cc. for the thymus of young rabbits, and 0.5 for the thymus of old ones. For the calf they give the value of 1.2 cc. Mendive and Deulofeu (5) found 0.5 to 0.6 mg. of vitamin C per gm. of tissue in the thymus glands of Argentine cattle.

The observation of von Euler and Klusmann that the vitamin C concentration in the thymus of the adult animal is much lower than that in the young was confirmed and extended by Yavorsky, Almaden, and King (6) who demonstrated that the vitamin C concentration, expressed in mg. per gm. of human thymus, fell from about 0.310 in infants from birth to 1 year of age to 0.190 in children from 1 to 10 years old, and to 0.046 in adults between 46 and 77 years old. These latter authors showed that the concentration of vitamin C decreasing with advancing age holds for a variety of other organs as well. Later in this paper it will be shown that

the decrease in the case of the thymus is due to displacement of tissue by fat and fibrous tissue rather than to an actual decrease in the concentration of the vitamin C in the glandular tissue itself.

The present study deals with the correlation of the vitamin C concentration of the bovine thymus with its histological structure at various ages, from the early fetus to the adult animal.

#### EXPERIMENTAL

In order that the results of the present work may be comparable with those of our previous investigations bovine tissues were employed throughout. The histological similarity between the bovine and human thymus was particularly advantageous. The glands were obtained directly from the killed animals, stored at  $-5^{\circ}$  as already described (1), and used for the titration of vitamin C within 1 day.

The micromethod employed previously (7) was used in the present case, since it offered advantages of greater simplicity and speed than was possible with the usual macroprocedure. Each c.mm. of standard 2,6-dichlorophenol indophenol solution used for the titration was equivalent to 0.125 microgram of vitamin C.

A cork borer having an internal diameter of 4.2 mm. was pushed through a block of frozen thymus tissue from 5 to 10 mm. thick, and the cylinder of tissue thus removed was pushed out of the cork borer on to the head of a freezing rotary microtome with the aid of a glass rod. A drop of physiological saline solution was used to make firm contact between the tissue and the freezing head. The sections were cut  $30\ \mu$  thick, and every 50th section was taken for analysis. The volume of each section was 0.415 c.mm. The homogeneity of the tissue is illustrated by the consistent titration values obtained. Five to ten sections were analyzed from each sample. The maximum deviation in the titration values for any sample was 0.20 c.mm. of the dye solution.

The block of tissue surrounding the hole left by the cork borer was fixed in Bouin's fluid, mounted in paraffin, sectioned  $6\ \mu$  thick parallel with the hole, and stained with Masson's trichrome stain (8); *e.g.*, iron hematoxylin, acid fuchsin with ponceau de xylydene, and light green.

The connective tissue and fat in the calf, and fetal thymus older than 4 months, are negligible; in the fetal organ younger than

4 months they may amount to 10 per cent, but in the adult animal they compose 30 to 95 per cent of the gland. The vitamin C concentration in connective tissue and fat is practically zero; hence the vitamin C concentration in the whole thymus of an adult animal would be very low, even though the concentration in the glandular portion might be appreciable. The following method was employed to determine the proportion of the glandular material to the connective tissue and fat in the thymus, so that the concentration of vitamin C in the glandular portion alone, as well as that in the whole organ, might be found.

By means of a microscope with a carbon arc source of illumination and a projecting mirror, the image of an entire stained section of thymus was thrown upon a sheet of graph paper  $22 \times 17$  inches (Eugene Dietzgen Company, No. 360). To obtain the lower power required to get the image of the entire section on the graph paper, neither an objective nor ocular was used on the microscope, but, instead, a biconvex lens having a diameter of 3 cm. and a focal length of 6 cm. was placed in the bottom of the microscope tube. With the microscope 8 feet from the graph paper, the area of the section was magnified 1600 times. The outlines of the glandular cell regions and those of the connective tissue and fat were traced on the graph paper with a pencil. Then the respective areas were cut out with scissors. The ratio of these areas is equal to the ratio of the volumes of the glandular and the connective and fat tissue in the section.

The areas may be determined in either of two ways. The squares on the graph paper may be counted, precaution being taken to include in any given area all whole squares and fractions one-half or greater; or the cut out graph paper of the two areas may be weighed separately on an analytical balance. Both methods have been tried and found to be in good agreement. In one section of steer thymus the graph paper weight of the glandular area was 6.8310 gm. and that of the fat and connective tissue area 6.1250 gm., giving 53 per cent by volume of glandular material. A different section from the same organ gave 4.5640 and 4.5428 gm., respectively, or 50 per cent. The square count for the latter section was 5952 and 5368 respectively or 53 per cent glandular material. It is essential for the weighing method that the graph paper be of uniform thickness and density. This was

tested by weighing equal areas of the paper, and it was found that the weight variation did not exceed 2 per cent. Because of greater simplicity and less tedium, the weighing method was adopted exclusively (Table I).

It was found that there was no histological difference in the thymus glands of the bull, steer, pregnant and non-pregnant cow, nor was there a difference between the thymus of a male and a female calf. Photomicrographs were taken of sections of thymus from an adult animal, a calf, an 8 month-old, and a 3½ month-old fetus to show the developmental histological changes that occur.

TABLE I  
*Per Cent of Glandular Tissue in Adult Bovine Thymus*

Gland No.	Animal	Weight of graph paper representing glandular por- tion	Weight of graph paper representing fat and connec- tive tissue portion	Glandular tissue
		<i>gm.</i>	<i>gm</i>	<i>per cent</i>
14	Bull	2.439	7.923	24
1	Steer	6.831	6.125	53
12	"	4.711	6.609	42
13	"	7.973	4.297	65
7	Cow, pregnant	5.299	11.238	32
9	" "	0.463	11.109	4
11	" "	6.748	5.597	55
17	" "	0.504	10.460	5
20	" "	1.088	13.591	7
22	" "	2.500	9.713	21
16	" non-pregnant	2.409	10.752	18

#### DISCUSSION

*Histological Changes of Thymus at Various Ages*—The thymus of the 3½ month bovine fetus is composed of large lobules separated by fine strands of connective tissue. The cortex and medulla are differentiating, but are not yet distinct. In the center of the lobules there are groups of large cells which later form the medulla; these are surrounded by the small lymphocyte-like cells of the cortex. Hassall's corpuscles are not distinguishable. Scattered throughout are numerous hematopoietic cells (Fig. 1). By the 8th month the differentiation of cortex and medulla is complete, and the lobules have increased slightly in size. The interlobular connective tissue is scant. The medulla is composed of large

reticular cells, and an occasional developing Hassall's corpuscle is present. Eosinophiles are scattered in both the cortex and medulla, and hematopoietic cells are also present (Fig. 2).

In the calf the lobules remain large and there is little interlobular connective tissue. The demarcation between cortex and medulla is sharp, both showing a compact arrangement of their cellular

TABLE II  
*Concentration of Vitamin C in Bovine Thymus at Various Stages of Development*

Gland No.	Animal	Average titration	Vitamin C per section	Vitamin C per gm. whole thymus	Vitamin C per gm. glandular tissue
		<i>c. mm. dye*</i>	<i>microgram</i>	<i>mg.</i>	<i>mg.</i>
14	Bull	0.41	0.05	0.12	0.50
1	Steer	0.77	0.10	0.23	0.44
12	"	0.92	0.11	0.28	0.67
13	"	1.21	0.15	0.36	0.55
7	Pregnant cow	0.58	0.07	0.17	0.53
9	" "	0.35	0.04	0.10	
11	" "	0.58	0.07	0.17	0.31
17	" "	0.15	0.02	0.04	
20	" "	0.28	0.03	0.08	
22	" "	0.54	0.07	0.16	
16	Non-pregnant cow	0.42	0.05	0.13	
2	Male calf	1.71	0.21	0.51	
3	Female calf	1.67	0.20	0.50	
19	Fetus, 20 cm. (3.6 mos.)	1.62	0.20	0.49	
21	" 30 " (4.4 " )	1.77	0.22	0.53	
6	" 35 " (4.8 " )	1.18	0.15	0.35	
10	" 40 " (5.2 " )	1.55	0.19	0.47	
8	" 66 " (7 " )	1.74	0.22	0.52	
18	" 80 " (8 " )	1.40	0.17	0.42	

\* These values have been obtained after subtraction of the blank value, 0.60 c.mm. The blank value equals the volume of dye required to bring the 50 c.mm. of 9 per cent acetic acid extracting solution employed to the same color as the standard (7).

elements. The Hassall's corpuscles are small and partly composed of a hyaline material. A fine branching, fibrous reticulum extends throughout the cortex and medulla and is related to the capillaries. Eosinophiles are present in the medulla. The development of the gland apparently has reached its maximum at this age (Fig. 3).

The thymus of the adult animal has undergone involution char-

acterized by replacement of the parenchyma by fat and connective tissue. The glandular elements are distributed as strands and masses throughout the fat. The distinction between cortex and medulla has disappeared, except for a slight increase in the reticular fibrous strands in the remnants of the medulla. The tissue is composed of uniform small cells resembling lymphocytes. There is an increase in the number of small blood vessels, and they show obliterative changes. The Hassall's corpuscles are small, and there are numerous concentric hyaline masses usually related to the reticular fibrous tissue (Fig. 4).

*Concentration of Vitamin C at Various Ages*—The concentration of vitamin C is given in Table II. Apparently there is no change in the concentration of the vitamin in the whole gland throughout the fetal and calf stages, the values varying between 0.35 and 0.52 mg. per gm. The cow and bull glands have a distinctly lower concentration of 0.04 to 0.17 mg. per gm., while that of the steer falls in the intermediate range of 0.23 to 0.36 mg. per gm. When, however, a correction is made for the fat and connective tissue, and the concentration is expressed in mg. of vitamin C per gm. of glandular cells, the values for all of the animals fall in the range, 0.31 to 0.67 mg. per gm. The corrections are based upon the data given in Table I. These corrections have been applied only in the case of those animals having 24 per cent or more glandular tissue in their thymus glands. When the percentage of glandular tissue is low, the error in the corrected value becomes prohibitively great. Thus if the concentration of the vitamin in the whole gland is 0.20 mg. per gm. and there is 80 per cent glandular tissue present, an increase of 2 in the latter figure would mean a difference of 3.4 per cent in the corrected value; the same variation at 25 per cent would produce a difference of 7.5 per cent; but the same variation at 10 per cent would make a difference of 16.5 per cent.

It would appear that the thymus, unlike most of the other organs, undergoes a decrease in concentration of vitamin C with increasing age merely because of displacement of glandular tissue by other tissues practically void of the vitamin, and not because of any significant decrease in concentration of the glandular tissue itself.

#### SUMMARY

The concentration of vitamin C in the bovine thymus has been determined for various stages of development from the early fetus

to the adult animal. The corresponding histological changes have been considered.

The concentration in the whole gland was found to remain fairly constant from the fetus of less than 4 months to the calf (0.35 to 0.52 mg. per gm.). The thymus of the steer had a lower concentration (0.23 to 0.36), and that of the cow and bull had a still lower value (0.04 to 0.17 mg. per gm.).

When a correction was applied for the fat and connective tissue present in the adult glands, it was found that the vitamin C concentration in the glandular cells was approximately the same for the adult animal as for the fetus or calf.

The authors wish to express their gratitude to Dr. E. J. Fallon, of the Department of Public Health, San Francisco, for furnishing the glands used in this investigation, and to Dr. G. Y. Rusk, head of the Pathology Department of the Mount Zion Hospital for his interest and suggestions during the course of this work.

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#### EXPLANATION OF PLATE 1

- FIG. 1. Photomicrograph of fetal bovine thymus gland, 3½ months; × 30.  
FIG. 2. Photomicrograph of fetal bovine thymus, 8 months; × 30.  
FIG. 3. Photomicrograph of calf thymus; × 30.  
FIG. 4. Photomicrograph of cow thymus; × 30







FIG. 1

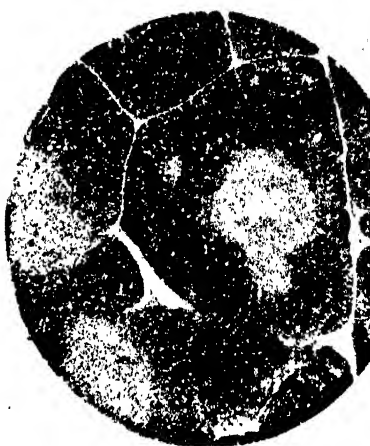


FIG. 2

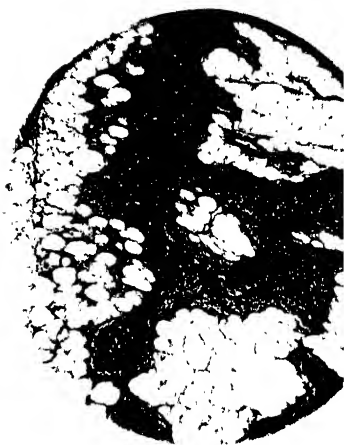


FIG. 4



## SYNTHETIC NUCLEOSIDES

### IV. THEOPHYLLINE-5-METHYL-*l*-RHAMNOFURANOSIDE

By P. A. LEVENE AND JACK COMPTON

(From the Laboratories of The Rockefeller Institute for Medical Research,  
" New York)

(Received for publication, February 12, 1936)

Inasmuch as the naturally occurring nucleosides possess the furanose structure,<sup>1</sup> work has been initiated in this laboratory aiming at the synthesis of nucleosides of this type. With classical methods the success of this undertaking depends upon the solution of two major problems: first, the preparation of bromoacetyl-furanopentoses; the second, the condensation of these with the silver salts of the purine or pyrimidine bases.

The present investigation is concerned with the second phase of the synthesis; namely, the problem of condensing an authentic 1-bromoacetylfuranopentose with silver theophylline. A very suitable sugar for the purpose of this investigation is *l*-rhamnose which readily forms a 2,3-isopropylidene derivative having a furanose structure. In this substance the hydroxyl in position (5) is readily blocked by methylation, thus permitting the preparation of the furanomethylpentose in which the possibility of a ring shift is excluded in subsequent operations.

Acetylation of 5-methyl-*l*-rhamnofuranose yields crystalline 5-methyl- $\alpha$ -triacetyl-*l*-rhamnofuranose<sup>2</sup> which is converted into the acetobromo compound in a manner similar to that employed by Schlubach and Wagenitz<sup>3</sup> for the preparation of  $\beta$ -acetobromogalactofuranose. The 5-methyl-acetobromo-*l*-rhamnofuranose obtained is extremely unstable, but rapid condensation with silver

<sup>1</sup> Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, **40**, 746 (1911).  
Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **94**, 809 (1931-32). Bredereck, H., *Ber. chem. Ges.*, **65**, 1830 (1932); **66**, 198 (1933).

<sup>2</sup> Levene, P. A., and Muskat, I. E., *J. Biol. Chem.*, **106**, 761 (1934).

<sup>3</sup> Schlubach, H. H., and Wagenitz, E., *Z. physiol. Chem.*, **213**, 87 (1932).

theophylline may be effected to yield theophylline-5-methyl-diacetyl-*l*-rhamnofuranoside as a sirup. Deacetylation of the acetate yields crystalline theophylline-5-methyl-*l*-rhamnofuranoside,  $[\alpha]_D^{20} = -46.3^\circ$  (in water), having a melting point of 212–213°.

The physical constants of the furanose nucleoside are quite different from those of the pyranose nucleosides, theophylline-4-methyl-*l*-rhamnopyranoside,  $[\alpha]_D^{25} = -71.8^\circ$  (in water), m.p. 179–180°, and theophylline-*l*-rhamnopyranoside,  $[\alpha]_D^{25} = -89.3^\circ$  (in water), m.p. 190–191°.

On the other hand, theophylline-4-methyl-*l*-rhamnopyranoside and theophylline-5-methyl-*l*-rhamnofuranoside showed great similarity in the rates of their hydrolysis with 0.03 *N* hydrochloric acid at 100° (Table III, Fig. 1). The fact that synthetic nucleosides of the pyranose ring structure are hydrolyzed as readily as certain naturally occurring nucleosides has been previously observed in this laboratory.<sup>4</sup> It is thus obvious that if this relation is true for all nucleosides, acid hydrolysis of similar naturally occurring products cannot furnish information in regard to the ring structure of the carbohydrate portion of the molecule.

This peculiarity in the behavior of the two nucleosides is conditioned by the nature of the aglucone and not by the differences in the structures of the carbohydrate residues, inasmuch as the two corresponding isomeric methylglycosides exhibit wide differences in the rates of their hydrolyses with 0.03 *N* hydrochloric acid at 100° (Table V, Fig. 1).

It thus can be seen from Tables III to V that in the case of the methyl and theophylline pyranoses the rate of hydrolysis is higher for the nucleoside whereas in the case of furanoses the rate of hydrolysis of the nucleoside is somewhat lower than that of the corresponding methylglycoside.

At this place, occasion is taken to state that the crystalline "5-methyl-acetobromo-*l*-rhamnose" with a melting point of 104–105°,  $[\alpha]_D^{25} = -184.2^\circ$  (in u.s.p. chloroform), previously reported,<sup>2</sup> was identical with an authentic specimen of 4-methyl-acetobromo-*l*-rhamnose with a melting point of 104–105°,  $[\alpha]_D^{20} = -183.9^\circ$  (in u.s.p. chloroform). Also that the theophylline nucleoside ob-

<sup>4</sup> Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, **65**, 463 (1925).

tained from the first substance (m.p. 178–179°,  $[\alpha]_D^{25} = -71.9^\circ$  in water) is identical with theophylline-4-methyl-*l*-rhamnopyranoside with a melting point of 179–180°,  $[\alpha]_D^{25} = -71.8^\circ$  (in water). Hence the previous conclusion regarding the structure of this nucleoside is now withdrawn.<sup>5</sup>

Incidentally, in connection with the work on the synthesis of the furanonucleosides, occasion was taken to clear up some points in the chemistry of certain rhamnose derivatives.

*α and β Forms of Monoacetone l-Rhamnose*—Freudenberg<sup>6</sup> has shown that in the condensation of *l*-rhamnose with acetone two isomeric monoacetone *l*-rhamnose derivatives containing the furanose ring structure are obtained with melting points and specific rotations of 87–89°,  $[\alpha]_D = +13^\circ \rightarrow +17.8^\circ$ ; and 79–80°,  $[\alpha]_D = +10.9^\circ \rightarrow +17.8^\circ$ , respectively. Since the isomers were found to mutarotate in aqueous solution to the constant value  $+17.8^\circ$ , it was assumed that they represented an  $\alpha, \beta$  pair. Owing to the similarity of the initial specific rotations, it has been impossible, however, to assign definite structures to the two substances. We have now found that the complete methylation of pure monoacetone *l*-rhamnose with a melting point of 92–93° with anhydrous Purdie's reagents gave monoacetone 5-methyl- $\beta$ -methylrhamnofuranoside,  $[\alpha]_D^{24} = +6.3^\circ$  (in water), whereas monoacetone *l*-rhamnose with a melting point of 79–80° yielded monoacetone 5-methyl- $\alpha$ -methylrhamnofuranoside,  $[\alpha]_D^{24} = -21.6^\circ$  (in water). From this result it is now possible to assign to monoacetone *l*-rhamnose with a melting point of 92–93° the  $\beta$  structure and to the lower melting isomer (m.p. 79–80°) the  $\alpha$  structure.

*5-Methyl-l-Rhamnofuranose*—The cleavage of 5-methyl-*l*-rhamnose phenylhydrazones<sup>6,7</sup> prepared from sirupy 5-methylrhamnose, with benzaldehyde<sup>8</sup> yields crystalline 5-methyl-*l*-rhamnofuranose. Since the substance exhibits no decided mutarotation, or any change in rotation is too rapid to be detected, the possibility that it exists in the free aldehydo structure was considered. Analysis

\* The results previously published represent preliminary conclusions which could not be corroborated at the time owing to the termination of Dr. Muskat's fellowship.

<sup>6</sup> Freudenberg, K., *Ber. chem. Ges.*, **59**, 836 (1926).

<sup>7</sup> Purdie, T., and Young, C. R., *J. Chem. Soc.*, **89**, 1194 (1906).

<sup>8</sup> Fischer, E., *Ann. Chem.*, **288**, 144 (1895).

of the absorption spectra of the substance in both water and dry dioxane showed no lines characteristic of the carbonyl group. It is thus evident that the substance is of a very stable furanose ring structure. On the other hand, 4-methyl- $\beta$ -*l*-rhamnopyranose mutarotates rapidly in water solution in a manner similar to that of  $\beta$ -*l*-rhamnose (Table I).

*5-Methyl-l-Rhamnose Phenyllosazone*—A statement was made by Purdie and Young<sup>7</sup> that a phenyllosazone of 5-methyl-*l*-rhamnopyranose was unobtainable. However, we have found it possible to prepare 5-methyl-*l*-rhamnose phenyllosazone when due precautions are taken, m.p. 123–124°,  $[\alpha]_D^{24} = +65.3^\circ$ , changing after 3 days in pyridine-ethanol (3:2) to  $[\alpha]_D^{24} = +44.4^\circ$ . This osazone is quite different from 4-methyl-*l*-rhamnose phenyllosazone, m.p.

TABLE I  
*Mutarotation of 4-Methyl- $\beta$ -l-Rhamnose*

$c = 4.020$  in water, 2.0 dm. tube.

$[\alpha]_D^{24}$	$t$	$[\alpha]_D^{24}$	$t$
degrees	min.	degrees	min.
+20 1	3	+4 4	15
+14 9	6	+1 7	20
+10 8	8	+0 5	25
+7 8	10	$\pm 0$ 0	30 (Constant)

162–163°,  $[\alpha]_D^{24} = +25.8^\circ$ , changing after 24 hours to  $[\alpha]_D^{24} = 0.0^\circ$  and after 2 weeks to  $[\alpha]_D^{24} = +14.3^\circ$  (in pyridine-ethanol (3:2)).

#### EXPERIMENTAL

*Monoacetone 5-Methyl- $\alpha$ - and  $\beta$ -Methyl-l-Rhamnopyranosides*—With slight modification, the two isomeric monoacetone *l*-rhamnose derivatives were methylated with Purdie's reagents in a manner similar to that described by Freudenberg.<sup>6</sup>

(a) 45 gm. of pure monoacetone *l*-rhamnose (m.p. 92–93°) were dissolved in 100 cc. of methyl iodide, containing 5 gm. of anhydrous sodium sulfate, and 100 gm. of silver oxide were added under vigorous stirring over a period of 4 hours at 50°. After the last addition stirring was continued for 1 hour. The product was freed of the reagents in the usual manner and subjected to

four further methylations in a similar manner. There were thus obtained 42.5 gm. of material with a boiling point of 58–60° at 0.3 mm.  $n_D^{25}$  1.4364. The specific rotation of the  $\beta$  isomer in water was

$$[\alpha]_D^{25} = \frac{+0.18^\circ \times 100}{2 \times 1.418} = +6.3^\circ$$

The composition of the substance agreed with that of a methyl methylhexomethylloside.

6.259 mg. substance: 13.055 mg. CO<sub>2</sub> and 4.885 mg. H<sub>2</sub>O

4.050 " " : 8.145 " AgI

C<sub>11</sub>H<sub>20</sub>O<sub>5</sub>. Calculated. C 56.86, H 8.70, OCH<sub>3</sub> 26.72

232.2 Found. " 56.88, " 8.73, " 26.54

(b) 60 gm. of monoacetone *l*-rhamnose (m.p. 79–81°) were dissolved in 130 cc. of methyl iodide and 130 gm. of silver oxide with 5 gm. of anhydrous sodium sulfate were added over a period of 4 hours under constant stirring at 50°. Following the last addition, stirring was continued for 20 hours at room temperature after which the reagents were removed in the usual manner. The product was subjected to a second methylation as described above, and then distilled at 0.3 mm. pressure. Yield 55 gm. B.p. 58–60° at 0.3 mm. The specific rotation in water was

$$[\alpha]_D^{25} = \frac{-0.61^\circ \times 100}{2 \times 1.414} = -21.6^\circ$$

The composition of the substance agreed with that of a methyl methylhexomethylloside.

5.203 mg. substance: 10.890 mg. CO<sub>2</sub> and 3.905 mg. H<sub>2</sub>O

4.990 " " : 10.020 " AgI

C<sub>11</sub>H<sub>20</sub>O<sub>5</sub>. Calculated. C 56.86, H 8.70, OCH<sub>3</sub> 26.72

232.2 Found. " 57.06, " 8.39, " 26.50

Due to the difficulty of obtaining monoacetone  $\alpha$ -*l*-rhamnose free from the  $\beta$  isomer, the specific rotation of monoacetone 5-methyl- $\alpha$ -methylrhamnofuranoside as given above may not represent the maximum value obtainable. Freudenberg reports  $[\alpha]_D = -32.5^\circ$  (in water) for the same product, which evidently was



obtained from a sample of monoacetone  $\alpha$ -*l*-rhamnose containing a smaller amount of the  $\beta$  isomer.

*5-Methyl-l-Rhamnose Phenylhydrazone*—The hydrolysis of monoacetone 5-methyl- $\alpha$ -methylrhamnofuranoside (55.0 gm.) with 1.5 per cent sulfuric acid solution was carried out, as previously described by Levene and Muskat,<sup>2</sup> to yield sirupy 5-methylrhamnose. In a similar manner monoacetone 5-methyl- $\beta$ -methyl-*l*-rhamnofuranoside yielded the same product which in neither case crystallized.

10.0 gm. of sirupy 5-methyl-*l*-rhamnofuranose were dissolved in 50 cc. of water, and 7 cc. of phenylhydrazine (1 mole) dissolved in 10 cc. of glacial acetic acid added. After thorough mixing the solution was allowed to stand 15 minutes at room temperature. At the end of this time, the hydrazone had crystallized. After two recrystallizations from absolute ethyl alcohol a constant melting point of 162–163° was obtained. Yield 4.5 gm. The specific rotation in dry pyridine was

$$[\alpha]_D^{24} = \frac{-0.45^\circ \times 100}{2 \times 1.222} = -18.4^\circ$$

changing after 18 hours to  $[\alpha]_D^{24} = \pm 0.0^\circ$ . Upon standing for 1 week the constant value of

$$[\alpha]_D^{24} = \frac{+0.20^\circ \times 100}{2 \times 1.222} = +8.1^\circ$$

was observed.

The composition of the substance agreed with that of a methyl hexomethylose phenylhydrazone.

3.602 mg. substance: 7.716 mg. CO<sub>2</sub> and 2.520 mg. H<sub>2</sub>O

3.200 " " : 7.40 cc. 0.01 N Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>

C<sub>13</sub>H<sub>20</sub>O<sub>4</sub>N<sub>2</sub>. Calculated. C 58.17, H 7.51, OCH<sub>3</sub> 11.59

268.16 Found. " 58.41, " 7.82, " 11.98

*Crystalline 5-Methyl-l-Rhamnofuranose*—Pure 5-methyl-*l*-rhamnose phenylhydrazone (3.0 gm.) was dissolved in 250 cc. of water heated to 95° and 3.3 cc. of benzaldehyde were added under vigorous stirring. The reaction was practically instantaneous, but stirring was continued for 45 minutes. The solution was then cooled, filtered, thoroughly extracted with ether, treated with charcoal, filtered, and concentrated under diminished pressure at

40° to a thick sirup. To remove the last traces of moisture the substance was taken up in alcohol (98 per cent) and the latter removed by distillation. The operation was repeated several times and finally the residue was taken up in benzene and the latter removed by distillation. This sirup was dissolved in ethyl acetate, filtered, and again concentrated under diminished pressure to a thick sirup. The last traces of this solvent were removed under a high vacuum at room temperature.

After standing in a vacuum desiccator over calcium chloride for several weeks, the sirupy 5-methyl-*l*-rhamnofuranose crystallized. After the second recrystallization from acetone, a constant melting point of 102–103° was obtained. Yield 1.2 gm. The specific rotation in water was

$$[\alpha]_D^{25} = \frac{-0.26^\circ \times 100}{2 \times 2.994} = -4.3^\circ$$

remaining constant.

The composition of the substance agreed with that of a methyl-hexamethylose.

4.591 mg. substance:	7.990 mg. CO <sub>2</sub> and 3.220 mg. H <sub>2</sub> O
3.390 " "	: 11.448 cc. 0.01 N Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub>
C <sub>7</sub> H <sub>14</sub> O <sub>6</sub> . Calculated.	C 47.16, H 7.92, OCH <sub>3</sub> 17.40
178.1 Found.	" 47.45, " 7.84, " 17.46

The substance is soluble in water, acetone, and pyridine, difficultly soluble in ether and benzene, and insoluble in petroleum ether. It reduces Fehling's solution strongly when heated and has a sharp, sweet taste.

*5-Methyl- $\alpha$ -Triacetyl-*l*-Rhamnofuranose*—42.5 gm. of sirupy 5-methyl-*l*-rhamnofuranose (see (a) above) were dissolved in 100 cc. of dry pyridine and 80 cc. of acetic anhydride were added at 0°. After standing overnight at room temperature, the mixture was poured with vigorous stirring into 2 liters of ice water. The sirup first separating crystallized completely upon standing a short time and was removed by filtration and dried. Yield 25 gm. After one recrystallization from methyl alcohol a constant melting point of 115–116° was obtained. The specific rotation in absolute methyl alcohol was

$$[\alpha]_D^{25} = \frac{-3.17^\circ \times 100}{2 \times 2.078} = -76.3^\circ$$

The aqueous filtrate remaining after removal of the crystalline 5-methyl- $\alpha$ -triacetyl-*l*-rhamnofuranose was thoroughly extracted with chloroform and the extract freed of pyridine in the usual manner. After drying over calcium chloride and concentrating under diminished pressure, a sirup was obtained which distilled under a high vacuum at 128–132° at 0.8 mm. Yield 15.0 gm. The specific rotation of this substance in absolute methyl alcohol was

$$[\alpha]_D^{25} = \frac{-0.90^\circ \times 100}{2 \times 3.306} = -13.6^\circ$$

The rotation confirms the opinion previously given that the sirupy 5-methyl-triacetyl-*l*-rhamnose is the  $\beta$  isomer.

TABLE II  
*Fractions Obtained on Methylation of 5-Methyl-*l*-Rhamnofuranose*

Fraction No.	B.p. at 0.3 mm.	Methoxyl content	Yield
	°C.	per cent	gm
I	75–80	38.62	2.0
II	85–95	32.29	2.5
III	100–135	30.12	2.5

*5-Methyl- $\alpha$ -Methyl-*l*-Rhamnofuranoside*—With slight modification 5-methyl- $\alpha$ -methyl-*l*-rhamnofuranoside was prepared according to the procedure of Purdie and Young.<sup>7</sup> 10 gm. of sirupy 5-methyl-*l*-rhamnofuranose were dissolved in 200 cc. of absolute methyl alcohol containing 1 per cent of dry hydrogen chloride. The solution was allowed to stand at room temperature for 20 hours after which it was heated on the water bath at 60° for 30 minutes. The solution was then cooled, the acid removed with excess silver carbonate, filtered, and concentrated under diminished pressure at 40° to a thick sirup. The product was then dissolved in dry ether, dried over calcium chloride, filtered, and concentrated under diminished pressure to a sirup which distilled under a high vacuum in three fractions as shown in Table II.

Fraction II crystallized after standing a short time and was freed of a small amount of Fraction I by pressing the crystals on a porous plate. The dry crystals, after the second recrystallization from petroleum ether (b.p. 30–40°), gave a constant melting

point of 59–60°. The specific rotation of the substance in water was

$$[\alpha]_D^{25} = \frac{-1.82^\circ \times 100}{2 \times 1.020} = -89.2^\circ$$

The composition of the substance agreed with that of a methyl methylhexomethylose.

4.434 mg. substance: 8.113 mg. CO<sub>2</sub> and 3.310 mg. H<sub>2</sub>O

3.400 “ “ : 21.22 cc. 0.01 N Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>

C<sub>8</sub>H<sub>16</sub>O<sub>6</sub>. Calculated. C 49.97, H 8.38, OCH<sub>3</sub> 32.40

192.1 Found. “ 49.90, “ 8.35, “ 32.27

The substance does not reduce boiling Fehling's solution, but after hydrolysis with dilute acid it reduces strongly upon heating. It is soluble in all the ordinary organic solvents and water.

*5-Methyl-l-Rhamnose Phenyllosazone*—1.0 gm. of 5-methyl-l-rhamnose phenylhydrazine (m.p. 162–163°) was dissolved in 25 cc. of 50 per cent acetic acid and 0.73 cc. (2 moles) of phenylhydrazine dissolved in 3 cc. of glacial acetic acid added. The solution was heated on the water bath for 10 minutes at 60° after which it was allowed to stand overnight at room temperature. The osazone crystallized from solution in long needles which after filtering and recrystallizing from 50 per cent methyl alcohol solution gave a melting point of 123–124°, unchanged by further recrystallizations. Yield 0.8 gm. The specific rotation of the substance in dry pyridine-absolute ethyl alcohol (3:2) was

$$[\alpha]_D^{25} = \frac{+0.47^\circ \times 100}{1 \times 0.72} = +65.3^\circ$$

changing after 3 days to

$$[\alpha]_D^{25} = \frac{+0.32^\circ \times 100}{1 \times 0.72} = +44.4^\circ$$

The solution then became too dark for further reading. The composition of the substance agreed with that of a methyl hexomethylose phenyllosazone.

4.006 mg. substance: 9.395 mg. CO<sub>2</sub> and 2.390 mg. H<sub>2</sub>O

4.700 “ “ : 7.944 cc. 0.01 N Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>

C<sub>10</sub>H<sub>24</sub>O<sub>8</sub>N<sub>4</sub>. Calculated. C 64.00, H 6.79, OCH<sub>3</sub> 8.70

356.2 Found. “ 63.95, “ 6.67, “ 8.73

*Theophylline-5-Methyl-l-Rhamnofuranoside*—5.0 gm. of dry 5-methyl- $\alpha$ -triacetyl-*l*-rhamnofuranose (m.p. 115–116°) were placed in a bomb tube and 8 cc. of pure dry hydrogen bromide condensed over it by cooling in a mixture of solid carbon dioxide-acetone. The tube was then sealed and allowed to come to room temperature slowly. The acetate had completely dissolved at the end of this time (about 15 minutes) and was allowed to stand 10 minutes longer at room temperature. The solution was then cooled in the cooling mixture, the tube was opened, and the hydrogen bromide was allowed to distil off at room temperature. The remaining sirup was dissolved in 50 cc. of dry toluene and the solvent removed under diminished pressure at 35°. The sirup was again taken up in dry toluene and concentrated rapidly under diminished pressure at 35°. The sirup, free of hydrogen bromide, was again taken up in 80 cc. of dry toluene and 6 gm. of dry, finely powdered silver theophylline were added to the solution. The mixture was shaken vigorously for 15 minutes at room temperature. The condensation was then complete. The solution was allowed to stand stoppered overnight, however, after which the silver salts were removed by filtration and thoroughly washed with dry toluene. The filtrate was then concentrated under diminished pressure to a thick sirup which was dissolved in a small volume of dry toluene and allowed to stand overnight in the cold. The small amount of theophylline separating was filtered off and the solution again concentrated under diminished pressure to a thick sirup which could not be induced to crystallize.

The sirups obtained from two runs were combined and dissolved in 300 cc. of dry methyl alcohol and 12 cc. of 0.45 *N* barium methylate solution added at 0°. The solution was allowed to stand 20 hours in the refrigerator after which the barium was exactly neutralized with 0.1 *N* sulfuric acid solution, treated with charcoal, and filtered. The clear filtrate was then concentrated under diminished pressure to a thick sirup, the last traces of water being distilled out by the repeated addition of absolute ethyl alcohol followed by dry benzene. The sirup was then dissolved in warm ethyl acetate, treated with charcoal, and filtered. Upon cooling, the nucleoside crystallized in long needles and was removed by filtration. After a second recrystallization from ethyl

acetate the constant melting point of 212–213° was obtained. Yield 1.1 gm. The specific rotation of the substance in water was

$$[\alpha]_D^{25} = \frac{-0.95^\circ \times 100}{2 \times 1.026} = -46.3^\circ$$

The composition of the substance agreed with that of a theophylline-methylhexomethylloside.

4.627 mg. substance:	8.385 mg. CO <sub>2</sub> and 2.500 mg. H <sub>2</sub> O
7.900 " " "	5.500 " AgI
3.390 " " "	0.488 cc. N <sub>2</sub> at 27° and 768.5 mm.
C <sub>14</sub> H <sub>10</sub> O <sub>6</sub> N <sub>4</sub> .	Calculated. C 49.40, H 5.88, N 16.46, OCH <sub>3</sub> 9.11
340.1	Found. " 49.41, " 6.04, " 16.55, " 9.19

The substance does not reduce boiling Fehling's solution, but after hydrolysis with dilute acid gives a strong reduction upon heating. The substance is insoluble in ether and petroleum ether, but very soluble in ethyl alcohol, methyl alcohol, and water.

*4-Methyl-Acetobromo-l-Rhamnose*—(a) 10 gm. of 4-methyl-triacetyl-rhamnopyranose were converted into 4-methyl-acetobromo-*l*-rhamnose (6.0 gm.) according to the procedure of Levene and Muskat.<sup>9</sup> The substance as obtained pure after the second recrystallization from dry ether gave a melting point of 104–105°. The specific rotation of the substance in U.S.P. chloroform was

$$[\alpha]_D^{25} = \frac{-7.55^\circ \times 100}{2 \times 2.052} = -183.9^\circ$$

(b) In a previous communication<sup>10</sup> we have shown that the supposed pure monoacetone methyl-*l*-rhamnofuranoside prepared according to the procedure of Levene and Muskat<sup>2</sup> was contaminated with monoacetone methyl-*l*-rhamnopyranoside, at times to the extent of 30 to 40 per cent. Accordingly, when this mixture of products is completely methylated, hydrolyzed, and acetylated, a mixture of 4- and 5-methyl-triacetyl-*l*-rhamnoses is obtained. Further, treatment of the mixed acetates with hydrogen bromide-glacial acetic acid yields a mixture of 4- and 5-methyl-acetobromo-*l*-rhamnoses. The former, being easily crystallizable, may be separated from the latter.

<sup>9</sup> Levene, P. A., and Muskat, I. E., *J. Biol. Chem.*, **105**, 431 (1934).

<sup>10</sup> Levene, P. A., and Compton, J., *J. Am. Chem. Soc.*, **56**, 2653 (1935).

5 gm. of mixed 4- and 5-methyl-triacetyl-*l*-rhamnose<sup>2</sup> were dissolved in 25 cc. of glacial acetic acid-HBr mixture (saturated at 0°) at 0° and allowed to stand 30 minutes. The acetic acid and hydrogen bromide were then removed as previously described and the sirup finally obtained was dissolved in 10 cc. of dry ether and cooled in an ice bath. Crystallization of the 4-methyl-acetobromo-*l*-rhamnose was complete after a few minutes and it was removed by filtration. After the second recrystallization from dry ether a constant melting point of 104–105° was obtained. A mixed melting point of this material and an authentic specimen of 4-methyl-acetobromo-*l*-rhamnose showed no depression. The specific rotation in U.S.P. chloroform was

$$[\alpha]_D^{25} = \frac{-3.70^\circ \times 100}{1 \times 2.008} = -184.2^\circ$$

*Theophylline-4-Methyl-β-l-Rhamnopyranoside*—(a) 2.3 gm. of 4-methyl-acetobromo-*l*-rhamnose (m.p. 104–105°) were dissolved in 50 cc. of dry xylene and 2.5 gm. of dry silver theophylline added. The mixture was thoroughly stirred and heated to 80–90° for 4 hours on an oil bath. At the end of this time the solution was free of bromine and the silver salts were filtered off and thoroughly washed with xylene. The filtrate was concentrated under diminished pressure to a thick sirup which was then dissolved in 10 cc. of dry benzene and allowed to stand in the cold overnight. The small amount of theophylline separating was filtered off and the solution concentrated to a thick glassy solid. The acetylated nucleoside could not be crystallized so it was necessary to deacetylate.

The diacetate obtained above was dissolved in 60 cc. of absolute methyl alcohol and 3 cc. of 0.45 N barium methylate solution were added at 0°. The mixture was allowed to stand 20 hours in the refrigerator, after which the barium was exactly precipitated with 0.1 N sulfuric acid solution. The solution was then treated with charcoal, filtered, concentrated under diminished pressure at 40° to a thick sirup, and dried by the repeated addition and distillation of absolute ethyl alcohol followed by dry benzene. The product soon crystallized spontaneously and after one recryst-

tallization from ethyl acetate gave the constant melting point of 179–180°. Yield 0.8 gm. The specific rotation in water was

$$[\alpha]_D^{25} = \frac{-1.49^\circ \times 100}{2 \times 1.038} = -71.8^\circ$$

The composition of the substance agreed with that of a theophylline-methylhexomethyloside.

4.532 mg. substance: 8.220 mg. CO<sub>2</sub> and 2.430 mg. H<sub>2</sub>O

3.980 “ “ : 2.760 “ AgI

3.144 “ “ : 0.454 cc. N<sub>2</sub> at 26° and 762 mm.

C<sub>14</sub>H<sub>20</sub>O<sub>6</sub>N<sub>4</sub>. Calculated. C 49.40, H 5.88, N 16.46, OCH<sub>3</sub> 9.11

340.1 Found. “ 49.46, “ 5.99, “ 16.46, “ 9.11

The substance does not reduce boiling Fehling's solution but after hydrolysis with dilute acid gives a strong reduction upon heating. The substance is soluble in ethyl acetate, pyridine, benzene, and water; insoluble in ether and petroleum ether.

(b) For further proof that the acetobromo compound obtained from a mixture of 4- and 5-methyl-triacetyl-*l*-rhamnose was 4-methyl-acetobromo-*l*-rhamnose, the product thus obtained was condensed with silver theophylline as described above to yield theophylline-2,3-diacyetyl-4-methyl-*l*-rhamnopyranoside as an amorphous solid. Deacetylation of 2 gm. of this material with barium methylate in absolute methyl alcohol yielded 1.4 gm. of theophylline-4-methyl-*l*-rhamnopyranoside, m.p. 178–179°. A mixed melting point of this material with an authentic specimen of 4-methyl-*l*-rhamnopyranoside showed no depression. The specific rotation in water was also identical with that of theophylline-4-methyl-*l*-rhamnopyranoside.

$$[\alpha]_D^{25} = \frac{-1.48^\circ \times 100}{2 \times 1.028} = -71.9^\circ$$

*4-Methyl-β-Methyl-l-Rhamnopyranoside*—4.0 gm. of 4-methyl-acetobromo-*l*-rhamnose (m.p. 104–105°) were dissolved in 100 cc. of absolute methyl alcohol and 8 gm. of silver carbonate were added at 0°. After shaking the material for 1 hour at room temperature the reaction was complete and the silver salts were removed by filtration and thoroughly washed with methyl alcohol. The fil-



trate was concentrated under diminished pressure to a thick sirup which distilled under a high vacuum at 105–106° at 0.3 mm. Yield 1.8 gm. No attempt was made to characterize further the 4-methyl-2,3-diacetyl- $\beta$ -methyl-*l*-rhamnopyranoside thus obtained.

1.8 gm. of 4-methyl-2,3-diacetyl- $\beta$ -methyl-*l*-rhamnopyranoside were dissolved in 50 cc. of absolute methyl alcohol and 3 cc. of 0.45 *N* barium methylate solution were added at 0°. The solution was allowed to stand for 20 hours in the ice box, after which the barium was precipitated with an equivalent of 0.1 *N* sulfuric acid solution, treated with charcoal, and filtered. The filtrate was concentrated under diminished pressure to a thick sirup which was dissolved in ethyl acetate, filtered, and again concentrated to a thick sirup. The product distilled at 104–105° at 0.3 mm. Yield 1.1 gm. The specific rotation in water was

$$[\alpha]_D^{24} = \frac{-0.36^\circ \times 100}{2 \times 1.288} = -13.9^\circ$$

The composition of the substance agreed fairly well with that of a methyl methylhexomethylloside.

5.625 mg. substance: 10.410 mg. CO<sub>2</sub> and 4.130 mg. H<sub>2</sub>O

3.500 " " : 21.068 cc. 0.01 *N* Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>

C<sub>6</sub>H<sub>14</sub>O<sub>6</sub>. Calculated. C 49.97, H 8.38, OCH<sub>3</sub> 32.40

192.1 Found. " 50.46, " 8.21, " 31.12

*4-Methyl- $\alpha$ -Methyl-*l*-Rhamnopyranoside*—10 gm. of 4-methyl-triacetyl-*l*-rhamnopyranose, deacetylated according to the procedure of Levene and Muskat,<sup>9</sup> yielded 3.5 gm. of crystalline 4-methyl- $\beta$ -*l*-rhamnose (m.p. 125–126°) and 3.2 gm. of sirupy 4-methyl- $\alpha$ -*l*-rhamnose. The sirup in which the  $\alpha$ -isomer predominates was used for the preparation of the  $\alpha$ -glycoside as follows:

3.2 gm. of sirupy 4-methyl-*l*-rhamnose were dissolved in 100 cc. of absolute methyl alcohol containing 1 per cent of dry hydrogen chloride. The solution was then heated on the steam bath for 60 minutes. At the end of this time the initial specific rotation of  $[\alpha]_D^{24} = -13.1^\circ$  had changed to the constant value  $[\alpha]_D^{24} = -47^\circ$ . The acid was neutralized with excess silver carbonate and the filtrate concentrated under diminished pressure to a thick sirup which was dissolved in 100 cc. of dry ether, treated with

charcoal, filtered, and again concentrated to a thick sirup which distilled under a high vacuum at 104–105° at 0.3 mm. Yield 3.2 gm. The specific rotation in water was

$$[\alpha]_D^{25} = \frac{-1.54^\circ \times 100}{2 \times 1.534} = -50.2^\circ$$

The composition of the substance agreed with that of a methyl methylhexomethyloside.

4.414 mg. substance: 8.097 mg. CO<sub>2</sub> and 3.200 mg. H<sub>2</sub>O  
 3.375 " " : 21.012 cc. 0.01 N Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>  
 C<sub>8</sub>H<sub>16</sub>O<sub>8</sub>. Calculated. C 49.97, H 8.38, OCH<sub>3</sub> 32.40  
 192.1 Found. " 50.00, " 8.11, " 32.19

*4-Methyl-l-Rhamnose Phenyllosazone*—0.5 gm. of 4-methyl-β-l-rhamnose was dissolved in 15 cc. of water and 0.82 cc. (3 moles) of phenylhydrazine dissolved in 3 cc. of glacial acetic acid added. After heating on the water bath for 20 minutes, the solution was cooled, whereupon the osazone crystallized in long yellow needles and was removed by filtration. After one recrystallization from 50 per cent methyl alcohol, the light yellow needles gave a constant melting point of 162–163°. The specific rotation in dry pyridine-absolute ethyl alcohol (3:2) was

$$[\alpha]_D^{25} = \frac{+0.18^\circ \times 100}{1 \times 0.696} = +25.8^\circ$$

changing after 24 hours to  $[\alpha]_D^{24} = 0.0^\circ$ . After 2 weeks the rotation was

$$[\alpha]_D^{25} = \frac{+0.10^\circ \times 100}{1 \times 0.696} = +14.3^\circ$$

The composition of the substance agreed with that of a methyl hexomethylose phenylosazone.

4.455 mg. substance: 10.410 mg. CO<sub>2</sub> and 2.690 mg. H<sub>2</sub>O  
 5.435 " " : 9.14 cc. 0.01 N Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>  
 C<sub>10</sub>H<sub>14</sub>O<sub>5</sub>N<sub>4</sub>. Calculated. C 64.00, H 6.79, OCH<sub>3</sub> 8.70  
 356.2 Found. " 63.72, " 6.75, " 8.84

*Theophylline-β-l-Rhamnopyranoside*—With the use of barium methylate in absolute methyl alcohol as a deacetylating agent in

place of alcoholic ammonia as used by Fischer and von Foder,<sup>11</sup> it is possible to obtain theophylline- $\beta$ -*l*-rhamnopyranoside in a much higher state of purity.

4.0 gm. of theophylline-triacetyl- $\beta$ -*l*-rhamnopyranoside (m.p. 135–136°) were dissolved in 120 cc. of absolute methyl alcohol and 6 cc. of 0.45 *N* barium methylate solution were added at 0°. After standing for 20 hours in the ice box the barium was exactly precipitated with 0.1 *N* sulfuric acid solution. The solution was then concentrated under diminished pressure to a thick sirup at 40°. The sirup was dissolved in 20 cc. of absolute ethyl alcohol, treated with charcoal, and filtered. The clear filtrate was allowed to stand overnight in the cold, whereupon the nucleoside crystallized from solution and was removed by filtration. There were thus obtained 1.5 gm. of material with a melting point of 190–191° unchanged by further recrystallizations from ethyl alcohol. The specific rotation of the substance in water was

$$[\alpha]_D^{25} = \frac{-1.84^\circ \times 100}{2 \times 1.030} = -89.3^\circ$$

The composition of the substance agreed with that of a theophylline-hexamethylsulfate.

4.512 mg. substance: 7.900 mg. CO<sub>2</sub> and 2.260 mg. H<sub>2</sub>O

3.915 " " : 0.596 cc. N<sub>2</sub> at 25° and 752 mm.

C<sub>13</sub>H<sub>18</sub>O<sub>6</sub>N<sub>4</sub>. Calculated. C 47.83, H 5.52, N 17.17

326.1 Found. " 47.74, " 5.60, " 17.25

*Comparison of the Action of 0.03 N Hydrochloric Acid Solution on Theophylline Nucleosides and Methylglycosides of the Pyranose and Furanose Ring Structures in the Rhamnose Series*

The ease with which glycofuranosides are hydrolyzed with dilute acid in comparison with the more stable glycopyranosides has frequently been made use of in determining the ring structure of sugar derivatives. For the first time there are now available synthetic nucleosides of the furanose and pyranose ring structures and use has been made of these substances to determine whether the useful relation found in the case of glycosides could be extended to this class of substances. The data recorded in Tables III to V and Fig. 1 have been obtained in the following manner: 0.2000

<sup>11</sup> Fischer, E., and von Fodor, K., *Ber. chem. Ges.*, **47**, 1058 (1914).

TABLE III

*Hydrolysis of Theophylline-4- and 5-Methylrhamnosides with 0.03 N HCl at 100°*

Run No.	Theophylline-4-methyl-rhamnopyranoside		Theophylline-5-methyl-rhamnofuranoside		<i>t</i> min.
	$[\alpha]_D$ degrees	Hydrolysis per cent	$[\alpha]_D$ degrees	Hydrolysis per cent	
I	-71.8	0	-46.3	0	0
II			-17.2	64.2	25
III	-34.0	52.6			30
IV			-8.1	84.3	55
V	-4.9	93.1			90
VI			-1.0	100.0	105 (Constant)
VII	0.0	100.0			120 "

TABLE IV

*Hydrolysis of Theophylline-1-Rhamnopyranoside with 0.03 N HCl at 100°*

Run No.	$[\alpha]_D^{25}$ degrees	Hydrolysis per cent	<i>t</i> min.
I	-89.3	0	0
II	-39.9	52.7	30
III	-15.7	78.6	60
IV	+1.0	96.2	150
V	+4.0	100.0	180 (Constant)

TABLE V

*Hydrolysis of 4- and 5-Methyl- $\alpha$ -Methylrhamnosides with 0.03 N HCl at 100°*

Run No.	4-Methyl- $\alpha$ -methyl-rhamnopyranoside		5-Methyl- $\alpha$ -methyl-rhamnofuranoside		<i>t</i> min.
	$[\alpha]_D$ degrees	Hydrolysis per cent	$[\alpha]_D$ degrees	Hydrolysis per cent	
I	-50.2	0	-89.2	0	0
II			-38.9	58.5	5
III			-10.9	91.1	15
IV			-3.3	100.0	25 (Constant)
V	-45.6	9.1			30
VI	-24.4	51.3			180
VII	-9.8	80.4			390

gm. of the substance was dissolved in exactly 20.0 cc. of 0.03 *N* hydrochloric acid solution and the rotation immediately observed. The ground glass-stoppered flask was then tightly stoppered and

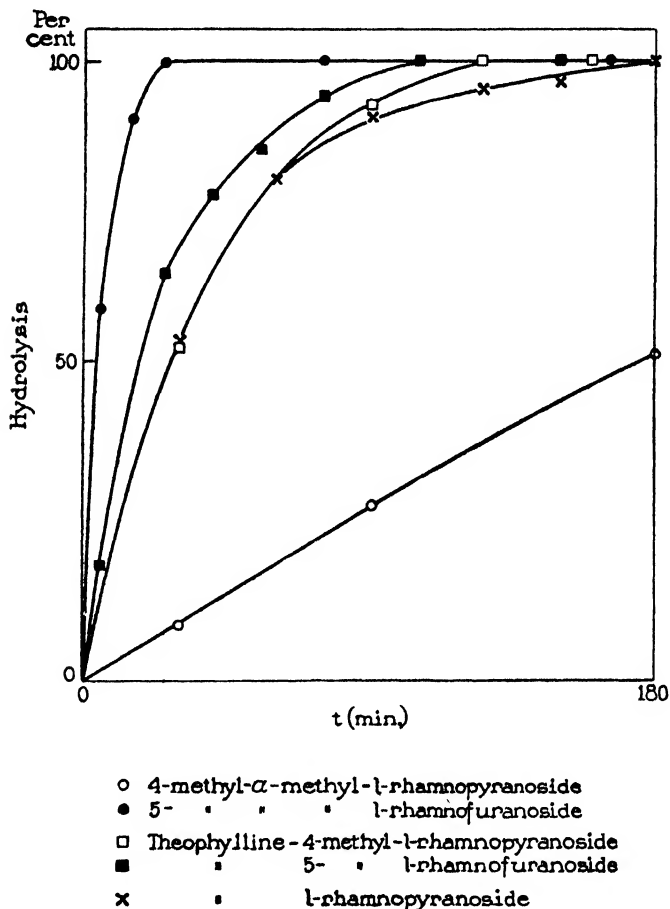


FIG. 1. The rate of hydrolysis of theophylline nucleosides and methylglycosides of *l*-rhamnose with 0.03 *N* HCl at 100°.

heated in a boiling water bath at 100° for the intervals of time specified, whereupon a portion of the solution was removed by a pipette and immediately cooled in an ice bath. The rotation of the solution was then immediately observed.

## THE FATTY ACIDS OF CHRYSALIS OIL

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The systematic researches of animal depot fats carried out by Hilditch, Tsujimoto, Klenk, and others have demonstrated that specific differences characterize the fats from marine, fresh water, and land sources. As far as land animals are concerned the investigations have dealt mainly with vertebrates. Our knowledge of the fats of invertebrates, such as insects, is rather limited. What is known has been largely derived from the study of the fat of a herbivorous insect, the chrysalis oil of the silk moth, *Bombyx mori*, which has been the subject of several qualitative analyses (1). In order to make an intelligent comparison between fats from insects and from other animals, a more quantitative study of the chrysalis oil seemed desirable, and it was the object of the present investigation to supply the necessary data.

These data, which are shown in Table III, demonstrate that the fatty acids of chrysalis oil contain four major components: palmitic, oleic, linoleic, and linolenic acids. As minor components stearic, palmitoleic, and unsaturated  $C_{20}$  to  $C_{22}$  acids were found. The main difference which distinguishes this insect fat from the fat of higher organized land animals is the relatively high content of linolenic acid.

### *Commercial Chrysalis Oil*

The chrysalis oil used in the present investigation was of the same stock as the oil which had been employed for the preparation of bombicysterol (2). Its properties are shown in Table IV. The oil was found to contain less than 1 per cent of acetone-in-

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soluble material. On the bottom of the flask containing the oil a white precipitate was observed, which disappeared on warming. On long standing the amount of precipitate increased considerably, reaching over 10 per cent of the oil.

*White Precipitate*—Piutti (3) claims that a white precipitate which he observed in chrysalis oil was stearic acid. In his opinion this acid owes its origin to a partial hydrolysis of the oil caused by the killing of the chrysalis with toxic gases. Stearic acid, however, as will be shown later, occurs in chrysalis oil only in small amounts, the bulk of the solid acids being palmitic acid. It seems, therefore, that Piutti's statement is erroneous.

When carefully purified, the white precipitate from commercial oil melted at  $67^{\circ}$  (stearic acid, m.p.  $70.5^{\circ}$ ). Its crystals were quite different from those of stearic acid. The substance was found to be neutral and to give palmitic acid and glycerol on saponification. From the yield of palmitic acid obtained it was concluded that the glyceride was a dipalmitate. The presence of a free hydroxyl group in the glyceride could be demonstrated by the formation of an acetate and a benzoate.

Of the two possible isomers of glyceryldipalmitate, which have been synthesized by Grün (4), only the symmetrical glyceride exhibits properties identical with those of the substance from chrysalis oil. One can, therefore, conclude that the white precipitate is glyceryl-1,3-dipalmitate.

This peculiar glyceride is absent in oils obtained from living chrysalises. It is apparently a product of decomposition caused either by the stifling of the cocoon or by aging. It could, however, not have been formed by a partial hydrolysis of any one of the eight glycerides which Suzuki and Yokoyama (5) have found in chrysalis oil, because none of them contains 2 molecules of palmitic acid to 1 molecule of glycerol.

*Isolation of Glyceryl-1,3-Dipalmitate*—The white precipitate contained in the oil was collected by centrifuging and washed with petroleum ether. The substance was then recrystallized four times from boiling petroleum ether and twice from alcohol. The glyceride which was obtained in a yield of 5 per cent crystallizes in small needles and melts at  $67^{\circ}$ .

*Saponification Number*—0.2961 gm. of substance required 10.37 cc. of 0.1 N KOH. Calculated for  $(C_{16}H_{31}CO_2)_2C_3H_5OH$ , 197.34; found, 196.6.

7.4 gm. were refluxed with 5 per cent alcoholic potassium hydroxide for 1 hour. The acids were then isolated in the usual way, giving 6.61 gm. of palmitic acid or 89.2 per cent. Calculated for a dipalmitate 90.13 per cent, for a tripalmitate 95.26 per cent, and a monopalmitate 77.57 per cent.

The palmitic acid after one recrystallization from alcohol melted at 62°.

0.4310 gm. of substance required 16.93 cc. of 0.1 N NaOH. Calculated for  $C_{16}H_{32}O_2$ , 256.25; found, 254.6.

When the substance was mixed with pure palmitic acid, no depression of the melting point could be observed.

From the neutral part of the saponification mixture 0.7 gm. of glycerol was obtained.

*2-Acetoglyceryl-1,3-Dipalmitate*—The glyceride was refluxed for 3 hours with 10 times its weight of acetic anhydride. After cooling the acetate was precipitated by the addition of water and recrystallized four times from alcohol. It crystallizes in the form of small needles, and melts at 47°. On cooling, the liquid substance solidifies at 29° and then melts constantly at 33°. The same behavior is shown by the synthetic substance.

*Analysis*—0.3036 gm. of substance required 14.74 cc. of 0.1 N KOH. Saponification number calculated for  $C_{37}H_{70}O_6$ , 275.7; found, 272.5.

*2-Benzoglyceryl-1,3-Dipalmitate*—Benzoyl chloride was added to a solution of the glyceride in pyridine. After 3 hours standing the mixture was poured into sufficient dilute  $H_2SO_4$  to neutralize the pyridine. The benzoate was precipitated as an oil which gradually solidified in the cold. It was filtered off, washed with water, and recrystallized four times from alcohol. The benzoate crystallizes in the form of long felty needles and melts at 44°.

*Analysis*—0.4298 gm. of substance after 3 hours refluxing with alcoholic potassium hydroxide had required 19.16 cc. of 0.1 N KOH. Saponification number calculated for  $C_{41}H_{72}O_2$ , 250.3; found, 250.1.

*The Fatty Acids*—The oil in which the white precipitate was redissolved by warming was saponified with alcoholic potassium hydroxide in an atmosphere of nitrogen. The non-saponifiable material was then removed by extraction with petroleum ether and the acids isolated in the usual way. They represented 91 to



92 per cent of the oil, were of a dark color, and showed an equivalent weight of 290 to 300 and an iodine number of 90 to 100.

The determination of solid acids by the Twitchell method met with difficulties. On addition of lead acetate to the alcoholic solution of the acids considerable amounts of a brown precipitate were formed, which proved to be almost insoluble in boiling alcohol and which interfered considerably with the determination. Since this behavior indicated the presence of oxidation products, the latter were removed with petroleum ether, in which they are rather insoluble.

For that purpose the acids were stirred for some time with 50 times their weight of petroleum ether. After standing for several hours the solution was then poured off the smeary brown oxidation products, and the solvents driven off under nitrogen. The remaining acids were redissolved in petroleum ether, and the solution to which some norit was added was kept standing overnight. After filtration and evaporation of the solvents a slightly yellow oil remained. The acids thus obtained represented 83.7 per cent of the oil and showed an equivalent weight of 284.1 and an iodine number of 122.1.

The acid mixture contained 23.85 per cent solid acids as determined by the Twitchell or 24.9 per cent as determined by the modified Bertram (6) method. For the isolation of larger quantities of solid and liquid acids the lead soap-ether method was used.

*Solid Acids*—The solid acids melted at 61–62° and showed an equivalent weight of 264.2 and an iodine number of 2.0.

The acids were esterified by refluxing them with absolute ethyl alcohol containing 5 per cent of concentrated sulfuric acid and the esters extracted with petroleum ether. 50 gm. of the ethyl esters were then subjected to a careful fractionation in the distilling apparatus described by Jantzen and Tiedtke (7).

The residue was distilled from a small Claisen flask, m.p. 35°, refractive index, 1.4352, acid m.p. 69–70°, equivalent weight 290.2 (0.3277 gm. required 10.17 cc. of 0.1 N NaOH).

The results of the fractionation (Table I) show that there can only be little if any of a  $C_{14}$  acid. The bulk of the acids is represented by a palmitic acid, but stearic acid also is present in considerable quantities. The presence of small amounts of an acid

higher than  $C_{18}$  is indicated by the high equivalent weight of the acid obtained from the last fraction. In agreement with the observation of Ueno and Ikuta (8) isopalmitic acid as found by Kawase *et al.* (9) could not be detected.

*Liquid Acids*—The liquid acids showed an equivalent weight of 285.5 and an iodine number of 161.0.

The liquid acids were hydrogenated in ether solution with platinum oxide as a catalyst. The hydrogenated acids melted at  $69^{\circ}$ . Equivalent weight 284.9, iodine number less than 2.

TABLE I  
*Fractionation of Ethyl Esters of Solid Acids*

Fraction No.	Ethyl esters			Acids					Theory	
	Yield	Refractive index at $42.5^{\circ}$	M.p.	M.p.	Equivalent weight	Substance	0.1 N NaOH		M.p.	Mol. wt.
	gm.		$^{\circ}C.$	$^{\circ}C.$		gm.	cc.		$^{\circ}C.$	
1	3.84	1.4310	24–25	60–61	257.2	0.6150	23.91		$C_{16}H_{32}O_2$ 62.5	256.3
2	4.33	1.4311	24–25	61.5						
3	4.67	1.4310	24.5	61.7						
4	2.33	1.4310	24.5							
5	2.62	1.4310	24–25							
6	2.31	1.4310	24.5	61.5	256.5	0.2578	10.05			
7	2.65	1.4310	24.5							
8	4.90	1.4310	24–25							
9	4.11	1.4310	24.5							
10	2.96	1.4312	24.7	62	257.5	0.5651	22.06			
11	1.40	1.4318	24–25	61–62	262.3	0.2355	10.88			
12	5.29	1.4338	25–28	65–67	272.1	0.5194	19.10		$C_{18}H_{34}O_2$ 70.5	284.3
13	2.25	1.4346	33	67.8	284.0	0.4340	15.25			
14	3.55	1.4349	34–35	69	287.0	0.3835	13.37			

The hydrogenated acids were ethylated and distilled as above.

Fraction 1 (Table II) indicates the presence of palmitic acid probably derived from the hydrogenation of palmitoleic acid. Fractions 2 to 10 contain ethyl stearate almost exclusively and Fraction 11 indicates the presence of acids higher than  $C_{18}$ .

In order to obtain more evidence for the presence of a palmitoleic acid, 250 cc. of the methyl esters of the mixed saturated and unsaturated acids were divided into four fractions by a fractional distillation in a Claisen flask. The first fraction (66 gm.)

was then subdivided into eleven fractions in the Jantzen and Tiedtke distilling apparatus. Fractions with similar refraction indices were united for further investigation.

Fractions 1 to 6 which contained the  $C_{16}$  acids weighed 30.9 gm. The iodine numbers observed ranged between 6 and 7, and the equivalent weights of the corresponding acids between 253.8 and 254.6. It is evident that the amount of palmitoleic acid present in the  $C_{16}$  fraction can only be rather small, certainly not over

TABLE II  
*Fractionation of Ethyl Esters of Hydrogenated Liquid Acids*

Fraction No.	Ethyl esters			Acids					
	Yield	Refractive index at 42.5°	M. p.	M. p.	Equivalent weight	Substance	0.1 N NaOH	Theory	
								M. p.	Mol. wt.
	gm.		°C.	°C.		gm.	cc.	°C.	
1	2.08	1.4319	32-33	63-64	268	0.1785	6.81	$C_{16}H_{32}O_2$	
2	11.24	1.4333	33.5	67-68	282.2	0.4305	15.14	62.5	256.3
3	8.72	1.4340	33.7	68-69	283.0	0.4856	17.16	$C_{18}H_{36}O_2$	
4	5.80	1.4343	33.7	68.7	284.5	0.6806	23.93	70.5	284.3
5	5.00	1.4343	33.7						
6	10.01	1.4343	33.7						
7	5.68	1.4343	33.7	69	284.4	0.2387	8.39		
8	4.48	1.4345	33.7						
9	4.00	1.4345	34						
10	1.32	1.4347	34	69-70	286.1	0.2950	10.32	$C_{20}H_{40}O_2$	
11	0.95	1.4364	35-37	70-72	301.0	0.1545	5.13	75.1	312.4

6 per cent. Attempts to isolate palmitoleic acid from the mixture failed.

The next two fractions (9.0 gm.) were mixtures of  $C_{16}$  and  $C_{18}$  acids and were not further investigated.

Fractions 9 to 11 (13.7 gm.) contained the  $C_{18}$  acids. The iodine numbers of these fractions ranged from 131 to 132. 4.3245 gm. of the ester mixture were saponified and the solid acids determined by Twitchell's method. 0.325 gm. or 7.94 per cent of stearic acid with a melting point of 69° and an equivalent weight of 284.7 was obtained.

*Fatty Acids of Freshly Prepared Chrysalis Oil*

Since the commercial chrysalis oil had shown signs of decomposition, fats extracted from living chrysalis of *Bombyx mori* were studied for comparison. Investigations were carried out with samples imported from Japan, China, and Italy and it was found that there are only insignificant differences in the composition of oils of different origins. As an example, the investigation carried out with the chrysalis of an Italian, yellow race of *Bombyx mori* will be described.

*Isolation of Oil*—739 chrysalises, weighing 1160 gm., were ground in a meat grinder and the pulp mixed with acetone. After a few hours the mixture was centrifuged, the supernatant liquid poured off, and the residue stirred with more acetone. After the procedure was repeated twice the acetone was replaced by ether and finally the residue was extracted with ether in a Soxhlet apparatus. The acetone was distilled off in a vacuum and the remaining aqueous liquid extracted with ether. All ether extracts were combined, dried, and evaporated to dryness. The entire procedure was carried out in an atmosphere of nitrogen.

The oil which weighed 58 gm. was of a clear yellow color. It did not deposit a precipitate on standing and was easily soluble in petroleum ether. Some of its properties are shown in Table IV.

By repeated treatment of a solution of the oil in ether with acetone, the oil was separated into an acetone-soluble and an insoluble fraction. The insoluble phosphatide fraction represented 8 per cent of the oil. It was a slightly yellow amorphous powder, the composition of which will be the subject of a separate investigation.

The acetone-soluble oil was saponified in the usual way. The soap solution was then repeatedly extracted with petroleum ether to remove the non-saponifiable fraction. This fraction, 2.5 per cent of the oil, possessed a deep yellow color quite different from the almost colorless non-saponifiable part obtained from commercial chrysalis oil. Otherwise their compositions are quite similar (2).

After acidification the soap solution yielded 94.08 per cent of a slightly yellow acid mixture which was completely soluble in petroleum ether. With Twitchell's method 4.6148 gm. gave

1.1394 gm. or 24.7 per cent of solid acids. Equivalent weight 264.1, iodine number less than 2, m.p. 61.5–62°.

The liquid acids isolated by the lead soap-ether method showed an iodine number of 163.4 and a thiocyanogen (Kaufmann) number of 123.3. From these figures it can be calculated that the mixture of unsaturated acids contains 50.49 per cent of oleic, 13.48 per cent of linoleic, and 35.93 per cent of linolenic acid.

4 gm. of unsaturated acids were placed in a small centrifuge bottle and dissolved in petroleum ether. To the solution, which had been cooled to  $-10^{\circ}$ , bromine was added until a slight yellow color remained. After 1 hour's standing the solution was centrifuged, the precipitate washed with ice-cold petroleum ether, and then extracted with ice-cold ether. 3.65 gm. of a white bromide remained, which were completely soluble in boiling benzene and which after one recrystallization from benzene melted at 178–180° (hexabromide of linolenic acid, m.p. 179°). From the hexabromide value and the iodine number of the unsaturated acids it can be calculated that the mixture contains 47.91 per cent of oleic, 18.56 per cent of linoleic, and 33.50 per cent of linolenic acid. These values agree reasonably well with those calculated from the iodine and thiocyanogen numbers. They differ, however, from the values reported by Kimura, who found 29.8, 29.2 per cent of oleic, 48.9, 35.9 per cent of linoleic, and 21.3, 34.9 per cent of linolenic acid. The first values were determined by the bromide method (10) and the second by the combination of the iodine and thiocyanogen numbers (11).

Mixtures of saturated and unsaturated acids obtained from different oils but having similar properties were united and methylated. 90 gm. were then divided into twenty-three fractions in the Jantzen and Tiedtke apparatus. The iodine numbers and melting points of the different fractions were determined, as were the amount of solid acids in the acid mixture after saponification, and the melting points and equivalent weights of the hydrogenated acids. The results of these observations are essentially the same as those obtained from commercial chrysalis oil. No definite evidence for a  $C_{14}$  acid could be found. The  $C_{18}$  acids show a slight degree of unsaturation, the iodine number being 6 to 7. The  $C_{18}$  acids showed an average iodine number of 137 and contained 8.06 per cent of stearic acid. The last two small fractions (1.55

gm.), having an iodine number of 138.6, gave after hydrogenation and saponification an acid which after one recrystallization from acetone melted at 72–73°. Its equivalent weight was 201.1 (0.3248 gm. required 10.79 cc. of 0.1 N NaOH). Here again the presence of  $C_{22}$  acids was indicated.

From all the results obtained it can be deduced that the average composition of the mixture of fatty acids from the chrysalis oil of *Bombyx mori* is as represented in Table III.

TABLE III  
Per Cent Composition of Acid Mixture

Saturated acids				Unsaturated acids				
$C_{14}$	$C_{16}$	$C_{18}$	$C_{20}-C_{22}$	$C_{18}$	$C_{18}$			$C_{20}-C_{22}$
					$-H_2$	$-H_4$	$-H_6$	
?	20	4	<1	2	35	12	28	1-2

*Chrysalis Oil of Tent Moth, Malacosoma americana*

For reasons of comparison the oil of a native American silk-spinning moth was investigated. Living tent moth chrysalises, collected during June and July, 1935, were extracted in the same manner as the chrysalis of *Bombyx mori*. From 870 chrysalises, representing 480 males and 390 females, and weighing 361 gm., 19 gm. of a clear yellow oil were obtained. For properties of this oil see Table IV.

18.2 gm. of oil gave 1.3 gm. or 7.1 per cent of an acetone-insoluble phosphatide. The acetone-soluble oil was saponified, giving 1.2 to 1.3 per cent of a yellow crystalline non-saponifiable fraction and 94.7 per cent of an acid mixture completely soluble in petroleum ether.

6.6324 gm. of mixed acids gave 2.9818 gm. of solid acids or 31.0 per cent. Equivalent weight 261.8, iodine number below 2.

The methyl esters of 4.8 gm. of solid acids were subjected to a fractional distillation. Of the four fractions obtained the first three contained methyl palmitate exclusively, while the fourth yielded an acid with a melting point of 65–66° and equivalent weight of 272. From the residue an acid was obtained which

TABLE IV  
*Comparison of Various Oils*

Sample	Density	Refractive index	Saponi- fication No.	Iodine No	Non- saponi- fiable material	Acid No.	Polen- ske No.	Reich- ert- Meissl No.	Hehner No.	Solid acids
										per cent
Various oils (1) *	D <sub>15.5</sub> 0.9280	20° 0.14757	189.9	105-132	1.6-10	8.5-62.8		3.2	94.5	23.8
	D <sub>40</sub> 0.9105	20° 0.14707								25
	D <sub>25</sub> 0.9230	25° 0.14720	189.9	109	1.5	7.1		3.0		24.4
Commercial oil.....	D <sub>25</sub> 0.9210	25° 0.14724	191.7	123	2.05	8	0.7	0.5	94.0	24.7
Fresh oil.....	D <sub>20</sub> 0.9259	20° 0.14707	192	133	2.08					25
Tussah oil (10, 11).....	D <sub>25</sub> 0.9231	25° 0.14712	191.5	106.2	1.25		0.7	0.5	94.7	30.1
Tent moth oil.....										

\* The figures in parentheses represent bibliographic reference numbers.

after two recrystallizations from acetone melted at 69°. Equivalent weight 282.1.

The liquid acids showed an iodine number of 150 and a thiocyanogen number of 111.3. Calculated, 57.45 per cent oleic acid, 19.7 per cent linoleic acid, and 22.85 per cent linolenic acid.

5.3 gm. of liquid acids were dissolved in 15 cc. of absolute alcohol and neutralized with 2 N lithium hydroxide solution. Sufficient water was then added to make the final volume 30 cc. After having been cooled for 12 hours, the salts of the lower unsaturated acids were filtered off and the filtrate evaporated to dryness. The residue was dissolved in a little water, dilute hydrochloric acid was added, and the acids were extracted with ether. 700 mg. of unsaturated acids having an iodine number of 208.8 were obtained. On hydrogenation stearic acid was formed with a melting point of 69–70° and equivalent weight of 285.1. The lower unsaturated acids obtained from the insoluble lithium salts were also hydrogenated. Here too stearic acid was obtained. The combined fractions of hydrogenated acids were methylated and the methyl esters were subjected to a fractional distillation. Four fractions were obtained all of which gave stearic acid on saponification. The material available for investigation was insufficient for a detection of minor components such as palmitoleic or C<sub>20</sub> to C<sub>22</sub> acids.

A comparison of the oils from the silk moth and tent moth demonstrates a great similarity.

#### SUMMARY

Samples of commercial chrysalis oil as well as the oil obtained from living chrysalises of several varieties of *Bombyx mori* have been investigated. It was found that commercial oil contained considerable quantities of a solid material which was identified as glyceryl-1,3-dipalmitate.

The mixture of fatty acids obtained from chrysalis oil consisted of 20 per cent palmitic, 4 per cent stearic, 2 per cent palmitoleic, 35 per cent oleic, 12 per cent linoleic, and 28 per cent linolenic acid, besides less than 1 per cent of saturated and 1 to 2 per cent of unsaturated acids containing more than 18 carbon atoms.

The composition of the chrysalis oil of the tent moth, *Malacosoma americana*, was very similar to that of *Bombyx mori*.



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## SOME CHEMICAL CONSTITUENTS OF FLOWERING DOGWOOD (CORNUS FLORIDA)\*

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In view of our interest in the general occurrence, distribution, and nature of plant coloring matters and of wax-like surface-covering constituents, including sapogenins, it was deemed desirable to investigate the nature of such substances occurring in dogwood flowers and bracts. Furthermore, this material afforded a means of developing a certain technique for the separation of the constituents of cuticle waxes which has since been applied in connection with studies on such important fruits as the apple, pear, cranberry, and grape. We were particularly fortunate in having available for such work a large quantity of dried material, representing nearly 175 pounds of fresh flowers and bracts.

The coarsely ground, air-dried material, weighing 16.3 kilos and corresponding to 79.6 kilos of fresh weight, was extracted first with ether and then with 95 per cent ethanol. The dark green residue (825 gm.) obtained from the ether extract was mixed with granulated pumice and extracted with petroleum ether (b.p. 30–60°) and finally with ether. The petroleum ether, ether, and alcoholic extracts were examined separately.

*Petroleum Ether Extract*—The dark green petroleum ether extract (330 gm.) was dissolved in ether and shaken with cold 25 per cent aqueous hydrochloric acid to remove colored impurities. The ether solution was then washed with water and the ether removed by distillation. The residue (214 gm.) was saponified with 3 per cent alcoholic potassium hydroxide, after which the mixture was shaken with ether to remove the unsaponifiable

\* Food Research Division Contribution No. 270.

material (67 gm.). Solid interfacial matter (18 gm.) was separated from the aqueous layer by filtration, and the solution of potassium soaps acidified. The free acids (128 gm.) were extracted with ether.

*Fatty Acids*—The fatty acids were separated by means of the lead salt-ether method into solid and liquid acid fractions, amounting to 16 and 89 gm., respectively.

*Palmitic and Stearic Acids*—A portion of the solid fatty acids was refluxed with phenacyl bromide according to the procedure of Rather and Reid (1) and the resulting esters were fractionally crystallized from alcohol. Two main fractions were obtained, melting at 63.2–64.7° and 51.8–52.4°, and corresponding in all respects to phenacyl stearate (m.p. 64–64.5°) and phenacyl palmitate (m.p. 52–52.5°). The mother liquors from the fractionation of the phenacyl esters were saponified and the free acids obtained therefrom added to the original solid acid fraction. Repeated crystallization of this material gave a product melting at 53.9–54.3° and setting to a solid at 52°. The melting point together with the neutralization value, which was found to be 207.8, indicated that the fraction consisted of a mixture of palmitic and stearic acids corresponding approximately to the eutectic containing 0.7 mole of palmitic acid (2).

*Linolenic and Oleic Acids*—The liquid fatty acids, dissolved in ether, were brominated in the usual manner at a temperature of –5° or lower. After standing several hours at 10° the hexabromides which separated were repeatedly crystallized from benzene. The linolenic hexabromide melted at 180–181° and gave on analysis<sup>1</sup> C 29.25, H 4.22, Br 63.91. Linolenic hexabromide,  $C_{18}H_{30}O_2Br_6$ , requires C 28.51, H 3.99, Br 63.28. No tetrabromide was detected in the residue obtained on evaporation of the filtrate from which the hexabromide had been removed. The residue was therefore debrominated with zinc in alcoholic solution and the recovered liquid acid subjected to distillation at 4 to 5 mm. pressure. The main fraction distilled at 164–165°, had an iodine number (Hanus) of 100.3, and therefore consisted principally of oleic acid.

<sup>1</sup> The writers are greatly indebted to Dr. R. T. Milner and Mrs. Mildred S. Sherman, Fertilizer Investigations, Bureau of Chemistry and Soils, for analytical results reported.

*Paraffin Hydrocarbon*—The unsaponifiable fraction was dissolved in hot alcohol, treated with charcoal, and filtered hot. The insoluble portion which separated on cooling consisted of a light yellow wax (58 gm.) melting at 65–67°. A portion was repeatedly fractionated, first from alcohol and then from petroleum ether-acetone solution. The fractions melting at  $65^{\circ} \pm 1^{\circ}$  were combined and treated in the usual manner with concentrated sulfuric acid to eliminate oxygenated impurities. The recovered hydrocarbon on crystallization from petroleum ether-acetone solution separated in large flat plates melting at 62–62.5° and setting solid at 61.2–60.9°. Transitions on heating were observed at 49–50° and 60–60.2° and again on cooling at 60.9–61.2° and 47–48°. x-Ray examination<sup>2</sup> of the unmelted material gave a spacing of  $39.2 \pm 0.2 \text{ \AA}$ . Analysis gave C 85.11, H 14.66. Nonacosane,  $C_{29}H_{60}$ , requires C 85.19, H 14.81. The observed values for the thermal and optical properties of the hydrocarbon isolated from dogwood do not agree exactly with corresponding values for any pure hydrocarbon or synthetic mixture studied by Chibnall and coworkers (3). The values indicate that the dogwood hydrocarbon is not a single compound but at least a ternary mixture in which nonacosane predominates. This conclusion is borne out by the enhanced value of the x-ray spacing and the low values of the melting and transition points.

*Phytosterol*—Another portion of the crude sterol-hydrocarbon fraction was dissolved in a mixture of hot alcohol and ethyl acetate. On cooling the bulk of the hydrocarbon separated and was removed by filtration. The mother liquors were concentrated to small volume, whereupon a second precipitate was obtained which was recrystallized from alcohol-ethyl acetate solution. The substance melted at 115–116° and responded to the well known tests for sterols. By converting it into the phthalyl derivative and fractionally recrystallizing the regenerated parent substance, a fraction was finally obtained (1 gm.) which melted at 124°. Analysis of the acetyl derivative, which melted at 110–111°, gave C 80.98, H 11.76.  $C_{26}H_{48}O \cdot COCH_3$  requires C 81.09, H 11.19. The sterol regenerated from its acetyl derivative melted at 122–

<sup>2</sup> Measurements of the crystal spacings were kindly made by Dr. Sterling B. Hendricks, Fertilizer Investigations, Bureau of Chemistry and Soils.

124° and gave C 79.72, H 12.01.  $C_{26}H_{43}OH \cdot H_2O$  requires C 79.92, H 11.88.

*Ether Extract*—The residue from the ether extract consisted of a yellowish, high melting resinous powder. It was digested several times with hot dilute sodium hydroxide solution and the insoluble portion filtered off. The soluble portion was discarded, since nothing of a crystalline nature could be isolated therefrom.

*Ursolic Acid*—The water-insoluble portion was dissolved in hot 95 per cent alcohol containing a slight excess of sodium hydroxide. The solution was filtered from insoluble material and an equal quantity of boiling water added, whereupon after evaporation of most of the alcohol, a copious precipitate of sodium salt occurred. After extraction of this material with ether to remove further impurities, it was recrystallized several times as before. The regenerated ursolic acid was further purified by fractional crystallization from 70 per cent alcohol, fractions melting below 275° being rejected. The remaining portions were combined and crystallized from 95 per cent alcohol, whereupon slender white needles or blades were obtained. These melted sharply at 283–284° and after being dried to constant weight at 140° gave on analysis C 78.89, H 10.60. Ursolic acid,  $C_{30}H_{48}O_8$ , requires C 78.88, H 10.60.

The methyl derivative, prepared in the usual manner with sodium ethoxide and methyl iodide, was finally crystallized several times from 50 per cent alcohol, after which it melted indefinitely between 110–115°, resolidified, and remelted at 170–171°. In the anhydrous state it gave on analysis C 79.02, H 10.72. Monomethyl ursolate,  $HO \cdot C_{29}H_{46} \cdot COO(CH_3)$ , requires C 79.08, H 10.71.

*Alcoholic Extract*—The original air-dried dogwood material after extraction with ether was extracted with hot 95 per cent alcohol in a continuous extractor of the Soxhlet type. The extract was partly concentrated and set aside for several months. A dark semicrystalline material which separated yielded 6 gm. of pure inositol and 2.4 gm. of scyllitol. The presence of these compounds has been reported elsewhere (4, 5). The remaining filtrate was freed of most of the alcohol, boiling water was added, and the filtered liquid treated successively with neutral lead acetate and basic lead acetate. The neutral lead precipitate was suspended in water and decomposed with hydrogen sulfide, the

mixture brought to a boil, and the lead sulfide filtered off. The orange-colored solution was then boiled with hydrochloric acid to hydrolyze the pigment glucoside and the resultant mixture shaken with ether. Upon evaporation of the ether, a crude residue was obtained which yielded a flavonol pigment and a water-soluble acid. The former was separated from the latter by boiling with water.

*Kaempferol*—The crude, insoluble yellow flavonol pigment was dried and acetylated by boiling 1 hour with acetic anhydride and anhydrous sodium acetate. The product was obtained from 70 per cent alcohol as colorless rods which melted at 116–120°, gradually resolidified, and again melted at 180–181°. Dried to constant weight at 130°, it gave on analysis C 60.65, H 3.90. Tetraacetylkaempferol,  $C_{15}H_6O_6(C_2H_3O)_4$ , requires C 60.79, H 3.96. The optical and crystallographic properties<sup>3</sup> of the acetyl derivative are given herewith. The crystals show straight and inclined extinction and negative elongation when examined with crossed nicols. The refractive indices as determined by the immersion method are  $\alpha = 1.500$  (not common),  $\beta =$  not determined,  $\gamma = 1.675$  (very common), both  $\pm 0.003$ . The double refraction is extremely strong ( $\gamma - \alpha = 0.175$ ).

Upon hydrolysis acetylkaempferol yields 63.2 per cent of free flavonol. This corresponds very closely to the theoretical yield (theory requires 63.00 per cent). The hydrolysis was carried out by boiling a short time in glacial acetic acid with a few drops of concentrated sulfuric acid. The free flavonol so obtained responded to tests for kaempferol and gave further confirmatory results on analysis, C 62.88, H 3.75. Kaempferol,  $C_{15}H_{10}O_6$ , requires C 62.92, H 3.52.

*Gallic Acid*—This acid was obtained from the neutral lead acetate precipitate of the dogwood alcoholic extract, after hydrolyzing the delead material and separating the kaempferol therefrom by boiling the residue from the ether-soluble portion with water. It was obtained from the aqueous portion by first treating with charcoal and then evaporating to small volume, whereupon on standing a yellowish white crystalline substance separated. The total yield amounted to 8 gm.

<sup>3</sup> All optical and crystallographic examinations were kindly made by Mr. G. L. Keenan of the Food and Drug Administration.

Identification was confirmed by conversion into its acetyl derivative, which was prepared in the usual manner with acetic anhydride. Recrystallization of the product from water yielded colorless rods which melted in the anhydrous state at 167–168°. Triacetylgallic acid prepared from an Eastman sample of pure gallic acid melted at 168–169°. Both samples yielded identical optical and crystallographic data, which are here summarized. In parallel polarized light (crossed nicols), the substance shows inclined extinction and positive elongation. The double refraction is extremely strong ( $\gamma - \alpha = 0.173$ ). In convergent polarized light (crossed nicols), the substance is shown to be biaxial, the two optic axes rarely appearing in the field simultaneously, but 2E is indicated to be rather large. Partial biaxial interference figures, however, are common, frequently showing one optic axis in the field. The refractive indices as determined by the immersion method are  $\alpha = 1.435$ ,  $\beta = 1.580$ ,  $\gamma = 1.608$ , all  $\pm 0.003$ .

*Quercetin*—The basic lead acetate precipitate obtained from the original alcoholic extract of the dogwood flowers and bracts was decomposed with hydrogen sulfide and the portion soluble in boiling water separated from the lead sulfide. The aqueous mixture was then boiled with 5 per cent hydrochloric acid to hydrolyze the pigment glucoside and the cooled solution extracted with ether. The crude residue obtained on evaporation of the ether, amounting to 4 gm., was acetylated and the product crystallized from 95 per cent alcohol, whereupon colorless rods were obtained. The substance melted at 194–196° and proved to be identical with pentaacetylquercetin. The substance showed straight extinction and negative elongation when examined with crossed nicols. The double refraction was very strong ( $\gamma - \alpha = 0.062$ ). The refractive indices as determined by the immersion method are  $\alpha = 1.580$  (lengthwise),  $\beta =$  not determined,  $\gamma = 1.642$  (crosswise), both  $\pm 0.003$ . On analysis the dried substance gave C 58.53, H 3.95. Pentaacetylquercetin,  $C_{15}H_5O_7(C_2H_3O)_5$ , requires C 58.57, H 3.93. On hydrolysis the acetyl derivative yielded the free pigment. It responded to tests for quercetin and gave further confirmatory results on analysis, C 59.50, H 3.41. Quercetin,  $C_{15}H_{10}O_7$ , requires C 59.59, H 3.34.

## SUMMARY

In addition to *inositol*,  $C_6H_{12}O_6$ , and *scyllitol*,  $C_6H_{12}O_6$ , which were previously reported, the following substances have been identified from the flowers and bracts of dogwood, *Cornus florida*: a mixture of hydrocarbons in which *nonacosane*,  $C_{29}H_{60}$ , predominates; a *phytosterol*,  $C_{26}H_{43}OH$ ; *palmitic*,  $C_{16}H_{32}O_2$ , *stearic*,  $C_{18}H_{36}O_2$ , *linolenic*,  $C_{18}H_{30}O_2$ , and *oleic acids*,  $C_{18}H_{34}O_2$ ; *ursolic acid*,  $HO \cdot - C_{29}H_{46} \cdot COOH$ ; *kaempferol*,  $C_{15}H_8O_2(OH)_4$ ; *gallic acid*,  $C_6H_2(OH)_3 \cdot COOH$ ; and *quercetin*,  $C_{15}H_8O_2(OH)_5$ .

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## THE ORIGIN OF NATURAL CRYSTALLINE UROBILIN (STERCOBILIN)\*

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Previous communications have dealt with the isolation and nature of crystalline urobilin and stercobilin (1-9). These substances, as obtained from human urine and feces respectively, were proved identical. It was further shown that crystalline mesobilirubinogen (urobilinogen) does not yield this type of urobilin upon exposure to air and light (8, 9). Instead of this a urobilin was isolated possessing a different crystal form and failing to yield the characteristic crystalline compounds of the natural substance; *i.e.*, the iron chloride molecular compound and the hydrobromide. This work has been confirmed and extended by Fischer, Halbach, and Stern (10). These investigators found that natural crystalline stercobilin is strongly levorotatory, while mesobilirubinogen and the urobilin prepared from it, previously spoken of as K-urobilin (9),<sup>1</sup> are optically inactive. Natural urobilin further differed from K-urobilin in that the melting point of the crystalline, free substance from acetone was 236°, while the free K-urobilin was found to melt at 190°.

From the foregoing it would appear that natural crystalline urobilin cannot be derived *in vitro* from mesobilirubinogen. Since the latter substance is identical with urobilinogen (11, 12), it remained to be determined whether a different urobilinogen is the parent substance of natural crystalline urobilin or whether the latter is derived from some other substance not having urobilinogen characteristics. The following studies were believed to be important to the solution of this question.

\* Aided by a grant from the research fund of the Graduate School, University of Minnesota.

<sup>1</sup> *Künstliches* urobilin.

1. Repetition of the isolation of a crystalline urobilin *in vitro* from mesobilirubinogen. This was desirable for the sake of further confirmation of results previously described (8, 9).

2. (a) Isolation of crystalline urobilinogen from urine in order to compare it again with mesobilirubinogen. (b) Isolation of crystalline urobilin from the mother liquor of the crystalline urine urobilinogen, after allowing it to develop urobilin characteristics by exposure to light and air.

3. Isolation of a urobilinogen from the feces in order to determine whether crystalline urobilin might be obtained from it after exposure to light and air.

4. Comparison of normal feces with feces during hemolytic jaundice as to total urobilinogen content estimated colorimetrically, and actual yield of crystalline stercobilin. This was desirable in order to decide whether stercobilin formation was more than proportionately increased in hemolytic jaundice.

#### EXPERIMENTAL

*Preparation of K-Urobilin from Mesobilirubinogen*—The method employed has been described previously (8, 9). The yield of K-urobilin from 75 mg. of crystalline mesobilirubinogen was but 2 mg. The crystals were identical in appearance with those already described. The substance again failed to yield a crystalline iron chloride molecular compound (7, 9) although this was readily obtained from a smaller amount of natural crystalline stercobilin. The absorption spectrum of solutions of these crystals does not differ from that of natural crystalline urobilin (9). The intensifying effect of the addition of water to an alcoholic solution, previously described for the natural substance (9), was also observed with K-urobilin.

*Isolation of Crystalline Urobilinogen from Urine*—The urine was obtained from a patient with hemolytic jaundice. The amount employed measured approximately 4 liters, requiring a period of 6 days to collect. The urine was collected in a brown glass bottle beneath a considerable amount of petroleum ether. At the time the isolation procedure was begun the urine exhibited a very intense Ehrlich reaction. The entire amount together with the petroleum ether was placed in a separatory funnel of 6 liters capacity. More petroleum ether was added until about 1 liter covered

the urine. The urine was next acidified with 100 cc. of glacial acetic acid and very gently mixed. After the carbon dioxide had entirely formed and disappeared, the urine and petroleum ether were mixed by repeated inversion for several minutes. The mixture was not shaken vigorously. Nevertheless a partial emulsion formed, most of which separated after a short period of standing; addition of a few cc. of 95 per cent alcohol furthered separation of the emulsion. The remainder of the emulsified portion was filtered with suction on a large Buchner funnel; this accomplished complete separation. The further treatment of the petroleum ether solution was as follows:

Petroleum ether  
↓  
5%  $\text{Na}_2\text{CO}_3$  (5 times)  
Acidified with 50%  $\text{H}_2\text{SO}_4$  until just blue to Congo paper  
↓  
 $\text{CHCl}_3$  (8 times)

Washed with water 2 times

After concentration *in vacuo* the  $\text{CHCl}_3$  solution was subjected to Fischer's procedure (11) for crystallizing mesobilirubinogen. A considerable amount of a very light yellow, almost white residue resulted when the final petroleum ether solution, obtained by this procedure, was concentrated to dryness *in vacuo*. This residue was dissolved in about 8 cc. of warm acetic ester, which was then concentrated to about 3 cc. After 5 hours in the ice box a considerable separation of light yellow, almost colorless crystals had occurred. The yield was 44 mg., 16 mg. of this amount being obtained by further concentration of the mother liquor. The substance was readily recrystallized from warm acetic ester. The crystals as first obtained are shown in Fig. 1. Recrystallization yielded small but perfect crystals such as are illustrated in Fig. 2.

The latter were examined by Professor Gruner of the Department of Geology, University of Minnesota, to whom the writer is indebted for the following report: "Small, probably monoclinic crystals. So far as I can determine, they appear to correspond entirely with the descriptions of hemibilirubin and mesobilirubinogen as given by Steinmetz in the reports of Fischer (11), Fischer and Meyer-Betz (12), and Fischer and Hess (13). Because of their small size, measurements could not be made." The first



FIG. 1. Crystalline urine urobilinogen as first obtained

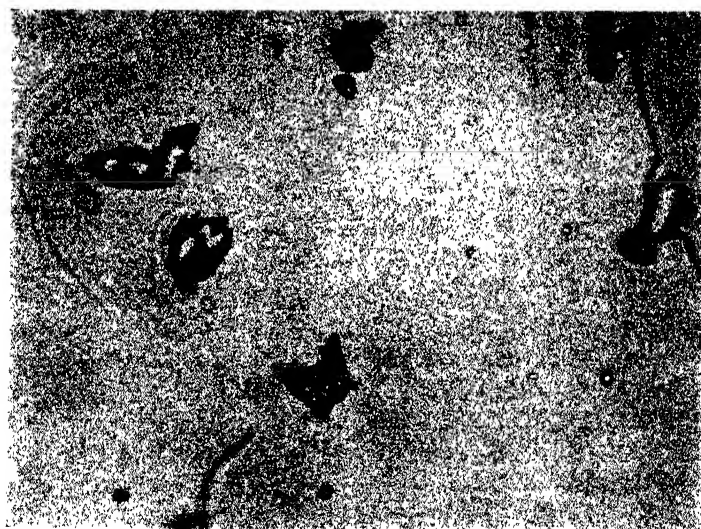


FIG. 2. Urine urobilinogen after recrystallization

crystals obtained melted at 193–196° with some shrinking above 190°; after recrystallization the melting point was 200–202° with slight shrinking above 192°. The melting point for mesobilirubinogen and urobilinogen was given by Fischer (14) as 201°. The crystals gave an intense Ehrlich reaction; the solution showed absorption at 568.3 to 542.2  $m\mu$ , maximum 558.9  $m\mu$ . A small amount of the crystalline substance was sent to Professor H. Fischer in Munich, for whose report, which follows, the writer acknowledges his gratitude.

"The absorption spectrum after carrying out the Ehrlich reaction is identical with that of mesobilirubinogen treated in the same manner. Crystals were submitted to Professor Steinmetz who stated that they were probably identical with those of urobilinogen as isolated by Fischer and Meyer-Betz (12), but that measurement could not be carried out because of the small size. After recrystallization from acetic ester-petroleum ether somewhat larger crystals were obtained; these were also believed by Professor Steinmetz to be probably identical with the urobilinogen of Fischer and Meyer-Betz, but were still too small for measurement."

*Isolation of Crystalline Urobilin from Mother Liquor of the Above Crystalline Urobilinogen*—After removal of the 44 mg. of crystalline urobilinogen mentioned above, the acetic ester mother liquor was allowed to dry. The residue remained exposed for several days to light and air and was then dissolved in 8 cc. of glacial acetic acid. This solution was allowed to stand in a lightly corked tube in the light for 2 weeks. At the end of this period its color was dark brown and the Ehrlich reaction negative. Green fluorescence with alcoholic zinc acetate solution was intense. The solution was poured into 4 volumes of chloroform and this was further treated in exactly the same manner as the first chloroform extract in the method previously described for the isolation of urobilin from the urine or stercobilin from the feces (3, 4). 17 mg. of crystals having the typical appearance of urobilin hydrochloride were obtained. From these the crystalline, free substance, hydrobromide, and iron chloride molecular compound were readily prepared according to the procedures previously described (2, 6, 7). The characteristic form of the crystals, particularly of the last two compounds, served to identify the substance with natural crystalline urobilin.

*Methods Employed in Attempt to Isolate Crystalline Urobilinogen from Feces, and in Isolating Crystalline Stercobilin from Various*

*Fractions Resulting during This Attempt*—202 gm. of feces were obtained over a 4 day period from a male patient, aged 20, who had suffered an injury to the left kidney with a resulting hematoma. The urobilinogen in this sample of feces was greatly increased. The average amount excreted per day during the 4 day period was found to be 1010 mg. A modification of a previously described method (15) was used for estimation of this amount. This modified procedure will be described in a separate communication.

About 2 gm. of the feces were employed in carrying out a test for occult blood with the benzidine method, which proved negative. The remaining 200 gm. were first ground in a mortar and washed repeatedly with petroleum ether, which was simply decanted in each instance. These washings gave only a weak Ehrlich reaction. The entire amount was next thoroughly ground with 1 liter of 5 per cent sodium carbonate in distilled  $H_2O$ . This mixture was at once poured into 1 liter of 8 per cent ferrous sulfate ( $FeSO_4 \cdot 7H_2O$ ) solution with constant shaking. Filtration was begun immediately and was complete at the end of 1 hour. The ferrous carbonate was employed at this stage to facilitate extraction of the urobilinogen. The character of the precipitate permits quicker filtration, and the urobilinogen is protected during its extraction by the mildly reducing action of the ferrous carbonate.

The alkaline filtrate was washed three times with petroleum ether without vigorous shaking. It was next acidified with glacial acetic acid and extracted three times with petroleum ether. A partial emulsion formed which was quickly separated by filtration with suction through a Buchner funnel. The combined petroleum ether solution gave a strong Ehrlich reaction. This solution was now subjected to the same procedure as was applied to the primary petroleum ether extract of the urine, mentioned above. Then with Fischer's method (11) the final  $CHCl_3$  solution was treated according to the following plan. It should be noted that precipitates and solutions left behind in this fractionation which gave either a relatively weak, or no Ehrlich reaction, but which obviously contained substances having stercobilin characteristics, were saved in order to determine whether crystalline stercobilin might be isolated from them. In the following plan these fractions are designated by Roman numerals.

$\text{CHCl}_3$ , concentrated *in vacuo* to 20 cc.

↓  
1 liter petroleum ether

Orange ppt. (Fraction I)

Concentrated *in vacuo* to dryness

Light yellow residue dissolved in 15 cc. warm ethyl acetate

Concentrated to 5 cc. (Ehrlich's reaction intense)

No crystallization after standing overnight at 4°

↓  
600 cc. petroleum ether

Light yellow precipitate  
Attempt to crystallize out  
of ethyl acetate unsuccessful  
Strong Ehrlich's reaction

Filtrate concentrated *in vacuo* to 10 cc.  
Separation of 130 mg. of almost white substance giving intense Ehrlich's reaction  
Could not be crystallized out of acetic ester

↓  
Combined in  $\text{CHCl}_3$

↓  
Combined  $\text{CHCl}_3$  solutions

↓  
5%  $\text{NaHCO}_3$

Washed with  $\text{CHCl}_3$ , 8 times

Acidified with 50%  $\text{H}_2\text{SO}_4$

↓  
Aqueous Fraction II

$\text{CHCl}_3$ , concentrated *in vacuo* to 8 cc.

↓  
500 cc. petroleum ether

↓  
Ppt. (Fraction III)

Concentrated *in vacuo* to dryness

Attempt to crystallize residue out of ethyl acetate unsuccessful

Dissolved in 25%  $\text{HCl}$

2 cc. of 5%  $\text{FeCl}_3$  in 25%  $\text{HCl}$  added

↓  
Filtrate

Voluminous brown granular ppt. similar in appearance to iron chloride double salt of mesobilirubinogen (Fischer and Niemann (16))

↓  
Washed with 25%  $\text{HCl}$  3 times

1%  $\text{NaOH}$

↓  
Ppt. of  $\text{Fe}(\text{OH})_3$

Filtrate acidified to Congo paper with 50%  $\text{H}_2\text{SO}_4$

↓  
Aqueous Fraction IV

$\text{CHCl}_3$ , (concentrated *in vacuo* to 5 cc.)

↓  
200 cc. petroleum ether (concentrated *in vacuo* to dryness)  
Almost white residue (intense Ehrlich's reaction)

↓  
3 cc. ethyl acetate (concentrated *in vacuo* to 1 cc.)  
No crystallization even after seeding with crystalline urobilinogen (from urine)



The dried residue was now subjected to the procedure described above by which crystalline urobilin was obtained from the mother liquor of the urine urobilinogen. In this way 13 mg. of crystals having the typical appearance of natural stercobilin hydrochloride were obtained. This material was identified by preparation of the crystalline hydrobromide and the iron chloride molecular compound.

Varying smaller amounts of crystalline stercobilin were likewise obtained from each of the Fractions I to IV in the accompanying diagram. To accomplish this, those of the fractions which were aqueous were acidified with hydrochloric acid, and the stercobilin hydrochloride was extracted with chloroform. Fractions consisting of precipitates were dissolved in 25 per cent HCl; after dilution with water the hydrochloride was extracted with chloroform. From this point the chloroform solutions were handled according to the method described previously (4). The crystalline iron chloride molecular compound was prepared in each instance for the sake of identification.

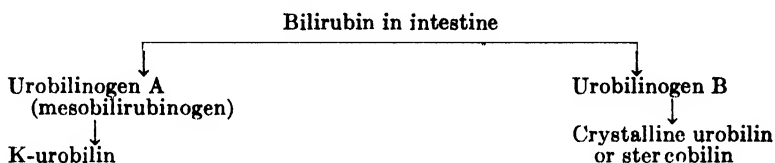
*Comparison of Normal Feces and Feces during Hemolytic Jaundice As to Total Urobilinogen Content and Yield of Crystalline Stercobilin*—The total amount of feces for 4 days was collected from a healthy male student 25 years old. This amount weighed 223 gm. (moist). The urobilinogen content was found to be 359.2 mg. The amount of crystalline stercobilin isolated was 73 mg. or 20.3 per cent of the estimated urobilinogen content.

The total amount of feces from a male patient, 18 years old, suffering from familial hemolytic jaundice, was collected for a period of 8 days. This weighed 920 gm. During the first 4 days the urobilinogen content was 4560 mg., and during the second 4 days 4880 mg., a total of 9440 mg. The amount of stercobilin isolated from the feces for 8 days was 1910 mg., or 20.1 per cent of the estimated urobilinogen content.

#### DISCUSSION

The results of the above experiments confirm those previously reported (8-10), which indicated that mesobilirubinogen does not yield natural crystalline urobilin or stercobilin. They further prove that the latter substances, already shown to be identical, may be obtained from a chromogen having urobilinogen charac-

teristics, occurring in both urine and feces. This chromogen undoubtedly differs from mesobilirubinogen or crystalline urobilinogen as isolated from urine by Fischer and Meyer-Betz (12), and again in the present investigation. Of distinct importance was the isolation of natural crystalline urobilin from a mother liquor which had already yielded crystalline mesobilirubinogen. These findings point to but one conclusion; *i.e.*, the existence in urine and feces of two urobilinogens. The following scheme may be used in further considering their relationship.



Urobilinogen B has not been obtained in crystalline form, probably because of its poor ability to crystallize. It was previously noted (4) that the leuco compound obtained by amalgam reduction of crystalline stercobilin could not be crystallized. This has been confirmed by Fischer, Halbach, and Stern (10). Probably for the same reason, K-urobilin has not been isolated from urine or feces, and can be crystallized only with difficulty after formation *in vitro*. There is every reason to believe that it accompanies the other, easily crystallized urobilin in nature. The failure to obtain mesobilirubinogen, or urobilinogen A, from the feces in contrast to the ease with which it may be isolated when present in comparable amount in the urine, suggests that this substance is more readily and completely reabsorbed from the bowel.

The above scheme assumes that urobilinogen B is derived from bilirubin. Direct proof of this is lacking, but the close similarity of the two urobilinogens as well as of their respective urobilins makes this most probable. The assumption is further supported by the recent observation of Fischer and associates (10) that crystalline stercobilin yields a porphyrin very similar spectroscopically to that obtained from bilirubin or mesobilirubinogen by the same treatment; *i.e.*, heating with glacial acetic and hydrobromic acids in a sealed tube at 180°.

Another possibility must be mentioned; *i.e.*, that urobilinogen B is a derivative of mesobilirubinogen by virtue of some process

peculiar to the chemical activity in the bowel. Two facts appear to be opposed to this theory: (1) the failure to obtain methyl-ethylmaleinimide by the nitric acid oxidation of stercobilin (1, 2, 10); (2) the failure by Fischer and coworkers (10) to obtain hematic acid from stercobilin following oxidation with chromic acid, instead of which succinic acid was isolated. Mesobilirubinogen yields both of these substances when oxidized by the same reagents (17).

The isolation of crystalline stercobilin from the various Fractions I to IV mentioned above is of importance, since it proves that stercobilin formation is not dependent upon any particular procedure employed, such as that previously used (4) in its isolation.

The amounts of stercobilin which may be isolated from the feces in hemolytic jaundice are relatively so large that the question arose as to whether they were not actually out of proportion to the increased blood destruction in this disease. That this is not the case is shown by the close agreement of yields of stercobilin in terms of per cent of total urobilinogen contents of feces in normal cases and in patients with hemolytic jaundice.

#### SUMMARY

1. Mesobilirubinogen, although identical with a urobilinogen occurring in the urine, is not the parent substance of crystalline urobilin or stercobilin as obtained from urine or feces. A different crystalline urobilin may be obtained *in vitro* from mesobilirubinogen. This urobilin probably occurs in nature but has not yet been isolated because of greater lability and poorer ability to crystallize.

2. The existence of a second urobilinogen in the feces and urine is indicated by the following: (a) The isolation from the feces of a non-crystalline chromogen possessing urobilinogen characteristics, which, after exposure to light and air, yielded natural crystalline stercobilin. (b) The isolation of natural crystalline urobilin from a mother liquor which had been allowed to develop urobilin characteristics by exposure to light and air. This mother liquor originally contained only chromogen, a part of which was isolated in the form of crystalline mesobilirubinogen.

3. The yield of crystalline stercobilin from feces in cases of

hemolytic jaundice is in the same proportion to the initial urobilinogen content as in normal feces.

*Addendum*—Recent studies, which have been reported since this paper was submitted for publication, suggest that natural crystalline urobilin may be derived from mesobilirubin. A bilirubinoid substance similar in constitution to mesobilirubin, but more closely resembling crystalline urobilin in physical properties, was synthesized by Siedel (18). Fischer and Halbach (19) were able to convert natural crystalline urobilin into glaucobilin. This substance, a derivative of mesobilirubin first described by Fischer, Baumgartner, and Hess (20), has not been found in nature. Fischer and Halbach's work is proof of the bile pigment nature of crystalline urobilin.

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## PREPARATION OF THE GONADOTROPIC HORMONE OF PREGNANT MARE BLOOD

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In recent years there has been an increasing interest in the gonadotropic material present in the blood and certain uterine tissues of the pregnant mare during a portion of the period of gestation. Unlike the gonadotropic materials found in human urine of pregnancy, the urine of menopause, and of women after ovariectomy, and the urine of persons suffering from certain tumors, such as chorioepithelioma and testicular teratoma, the gonadotropic substance of pregnant mare blood is rarely, if ever, found in the urine. The separation of the active material, which is most probably protein-like in nature, from the substances of the mare blood has naturally been more difficult than the preparation of gonadotropic material from urinary sources, which accounts for the greater study which the latter has received.

The determination of the relationship between the time of conception and the appearance of the hormone in the mare's blood stream, as well as the length of time that recognizable amounts of it may be detected there, has been the subject of several investigations. Cole and Hart (1, 2) and Küst (3, 4) have studied these relationships in farm horses, the breeding dates of which were known. Cole, Howell, and Hart (5) and Catchpole and Lyons (6) have investigated the relationship between the crown-rump length of the fetal colt in the Western range mare and the hormone content of the mare's blood. The most exhaustive studies have been those of Küst who has investigated the subject in several thousand farm mares of known breeding dates (7).

The chemical properties of the hormone have received but little study (8-10) and most of these investigations have employed the

whole serum or plasma or crude protein fractions obtained from the serum or plasma by precipitation methods. The crudity of these preparations greatly restricted the value of studies on the chemical nature of the active substance as well as investigations of its interesting biological properties. Clinical studies in man and more elaborate biological investigation involving costly laboratory animals (11) became feasible when Evans, Gustus, and Simpson (12) reported a method of obtaining the hormone in a more purified form from the acetone-dried protein material of the blood serum of pregnant mares. We have continued the study of this method and have extended it to the preparation of the hormone directly from the crude citrated plasma or serum of the mare.

The method employed by Evans, Gustus, and Simpson consisted of selective adsorption of the active material to aluminum hydroxide with subsequent elution. Our studies have shown that occasional samples of plasma may require much more adsorbent than plasma of equal potency from other mares, and for this reason it is advisable to make a series of preliminary adsorption tests on small quantities of diluted plasma to determine the amount of adsorbent needed to remove the activity. These occasional samples of plasma which prove difficult to adsorb have been conspicuous by the large number of corpora lutea which they develop in immature rats on injection of doses which ordinarily would cause purely follicular development. We are investigating this type of plasma in an attempt to throw light on this peculiarity.

Our experience with aluminum hydroxide adsorbents has shown the superiority of the Willstätter Type A over the Type B for use with this hormone. Evans, Gustus, and Simpson recommended both types and employed Type B in the experiment which they described. Although Type B is an excellent adsorbent for this purpose, it rapidly loses this property after a few months standing (13), while Type A retains its power to adsorb the hormone, practically unimpaired, for at least a year. We have made an intensive investigation of aluminum hydroxide adsorbents with the hope of finding a simpler and more convenient method of preparing a suitable adsorbent. The results of this study will be the subject of a future communication.

In routine work it has appeared advantageous to perform the adsorption in two steps, half of the required adsorbent being used each time. With some plasmas this leads to greater efficiency. It is also important to dilute the plasma sufficiently before attempting adsorption, since the efficacy of the adsorbent is much less in concentrated solutions of plasma.

The problem of preparing the purified hormone solution in a form suitable for clinical study has required an investigation of the applicability of various bacterial filters as a means of obtaining sterile solutions. Although the serum of the pregnant mare may be filtered through a Berkefeld filter candle without appreciable loss of potency ((9) p. 238), solutions of the purified hormone behave very differently. The Berkefeld and Seitz filters adsorb large quantities of the active material when filtration is

TABLE I  
*Preparation of Purified Hormone Solution for Clinical Study*

$\text{Na}_2\text{HPO}_4$ , anhydrous (98.8%)	$\text{H}_3\text{PO}_4$ (0.773 M)	$\text{Na}_2\text{HPO}_4$ $12\text{H}_2\text{O}$	$\text{H}_3\text{PO}_4$ (0.773 M)	pH of final solution	Freezing point of resulting solution
gm. per ml. solution	ml. per ml. solution	gm. per ml. solution	ml. per ml. solution		°C.
0.0160	0.0191	0.0410	0.0196	7.0	-0.556
0.0151	0.0404	0.0388	0.0415	6.5	-0.550
0.0135	0.0588	0.0347	0.0605	6.0	-0.549

performed with either an acid or alkaline solution. The Pasteur-Chamberland and Mandler filters adsorb the hormone from acid solutions but not from alkaline solutions (pH 8.3). Since the purified hormone has been found to be most stable in acid solutions (pH 5 to 6.5), we have prepared it for clinical study by filtering the solution through a Pasteur-Chamberland filter at pH 8.3 and acidifying the filtrate aseptically with sterile acid to pH 6.5. This may be readily accomplished by alkalizing the solution with disodium phosphate and acidifying it with phosphoric acid. The procedure may be carried on in such a way that the resulting solution is isotonic with blood. Table I, prepared from our experimental results, has served as a guide in calculating the amounts of disodium phosphate and phosphoric acid needed.

A description of a typical experimental run (Experiment H-1056) will make the procedure clear.



## EXPERIMENTAL

2400 ml. of plasma were diluted to 15 liters with water, adjusted to pH 3.5 with 10 per cent HCl, and clarified with the Sharples supercentrifuge. After checking the pH and readjusting it to 3.5, a suspension of aluminum hydroxide (Willstätter Type A) representing 14.2 gm. of  $\text{Al}_2\text{O}_3$  was added. The adsorbent was suspended in 600 ml. of water and added slowly while the diluted plasma was vigorously stirred. After the adsorbent was added, the stirring was continued for half an hour. The aluminum hydroxide was now collected by running the suspension through the Sharples supercentrifuge. After checking the pH of the clarified solution, the process of adsorption was repeated, the same amount of adsorbent and the same procedure being used. The combined aluminum hydroxide adsorbents carrying the active material were worked into a suspension in 2 liters of M/15 acetate buffer (pH 3.5), shaken for 1 hour, and the suspension was run through the supercentrifuge, recovering the adsorbent. This washing may be repeated and serves to eliminate material mechanically carried by the aluminum hydroxide.

The adsorbent was now worked into suspension in 150 ml. of water, the acidity neutralized with a few drops of 10 per cent ammonia, and an equal volume of 1 per cent ammonia added, and the suspension thoroughly shaken and allowed to stand overnight in the ice box to complete the elution of the active material. The following day the precipitate was separated in the supercentrifuge and the ammoniacal solution of the hormone was neutralized with dilute hydrochloric acid and dialyzed in bags made from No. 300 cellophane, first against running tap water at 12° for 30 hours, followed by 18 hours against distilled water in the ice box. The solution was now free from ammonium salts (Nessler's reagent) and showed a slight precipitate of globulin and aluminum hydroxide. This was centrifuged off and the solution frozen and thawed. This caused the separation of traces of aluminum hydroxide which were removed in the centrifuge. The perfectly clear and colorless solution (volume 340 ml.) was assayed. During the period of the assay the solution was kept frozen. The results showed practically complete recovery of the activity in the original serum.

The dilution necessary to bring the solution to the desired strength was computed from the assay (final volume 430 ml.) and the amount of disodium phosphate and phosphoric acid needed to make the solution isotonic with blood and have a pH of 6.5 was determined.

Accordingly, 6.43 gm. of  $\text{Na}_2\text{HPO}_4$  were dissolved in the hormone solution. The alkaline solution was then filtered through a Pasteur-Chamberland L-5 filter candle. The volume of the filtrate was observed and for each ml. of filtrate 0.0404 ml. of 0.773 M sterile phosphoric acid<sup>1</sup> was added aseptically. The adjusted solution was now put up in ampules and assayed. When kept in the ice box, its potency was retained for several months.

#### SUMMARY

The method of Evans, Gustus, and Simpson for the concentration of the gonadotropic hormone from solutions of the acetone-dried protein material of pregnant mare serum has been adapted to the preparation of this hormone directly from the citrated plasma of the mare.

The preparation of solutions of the hormone adapted to clinical study has been described.

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## THYROGLOBULIN STUDIES

### II. THE VAN SLYKE NITROGEN DISTRIBUTION AND TYROSINE AND TRYPTOPHANE ANALYSES FOR NORMAL AND GOITROUS HUMAN THYROGLOBULIN

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In the first of these studies (1) it has been shown that there is a definite variation in the thyroxine and non-thyroxine iodine (diiodotyrosine) content of thyroglobulin isolated from normal and goitrous human thyroids. This paper is a study on the same samples of thyroglobulin used in the previous study.<sup>1</sup> The Van Slyke nitrogen distribution method was used, supplemented with direct tyrosine and tryptophane analyses to determine whether there is a variation also present in the content of other amino acids present in the thyroglobulin molecule or whether the variation is peculiar only to the iodine-containing amino acids. The Cavett modification of the Van Slyke nitrogen distribution method (2) and the Folin and Marenzi tyrosine-tryptophane method (3) were used for the analyses. The presence of thyroxine and diiodotyrosine was found not to interfere with either the tyrosine or the tryptophane determinations.

The data presented in Table I are from Van Slyke nitrogen distribution and tyrosine and tryptophane analyses upon normal human thyroglobulin, the thyroids being obtained at autopsy. The data under Group I are from a composite of thyroids that were collected and classified according to the size of the acini. A few of these thyroids came from patients who had been pre-

<sup>1</sup> These samples have the same designations (group names and numbers) as in the previous study by Cavett, Rice, and McClendon, from which the values of thyroxine and diiodotyrosine are quoted. (The non-thyroxine iodine has been calculated to per cent diiodotyrosine.)



pared for operation by sterilizing the skin with iodine a few days before the autopsy. Later a second group (Group II) was collected, including only thyroids from patients where no iodine medication of any type had been used. The results of duplicate analyses are given for the members of Group II.

In Table II are the analyses of five samples of thyroglobulin obtained from five colloid glands. No preoperative iodine medi-

TABLE II  
*Van Slyke Nitrogen Distribution of Thyroglobulin from Colloid Goiters*

Gland No. ....	1	2	3	4	5	Average
Weight of gland, gm. ....	455	70	125	230	160	values for group
Per cent total N						
Amide N. ....	9 90	10 13	10 30	9 11	9 69	9 82
Humin " ....	3 44	3 33	3 49	4 08	4 11	3 69
Arginine N. ....	14 06	14 88	14 88	14 73	15 18	14 75
Cystine " ....	2 04	2 02	2 14	1 72	2 19	2 02
Histidine N. ....	3 11	3 11	2 95	2 83	2 92	2 98
Lysine N. ....	7 15	6 19	6 32	6 83	5 75	6 45
Filtrate amino N. .	56 31	55 26	57 13	56 28	57 47	56 49
" non-amino N ....	5 16	5 80	4 15	4 35	4 56	4 80
Per cent total protein						
Tryptophane. . . .	2 12	2 14	2 10		2 23	2 15
Tyrosine. ....	3 42	3 57	3 09		3 43	3 38
" + tyrosine equiv-						
alent. ....	3 44	3 58	3 11		3 47	3 40
Thyroxine. ....	0 0046	0 0061	0 0092	0 0105*	0 073	0 021
Diiodotyrosine. . .	0 046	0 031	0 04	0 057	0 066	0 048

\* Given as 0.105 in the paper by Cavett, Rice, and McClendon.

cation was employed with any of the individuals from whom the thyroids were obtained.

The data included in Table III are those obtained on the analysis of thyroglobulin from ten toxic adenomatous thyroids. Each individual from whom these thyroids were removed (except Gland 1) had received iodine in the form of Lugol's solution preoperatively.

Table IV presents data on the thyroglobulin from thyroids of persons with exophthalmic goiter. The individuals from whom

TABLE III  
*Van Slyke Nitrogen Distribution of Thyroglobulin from Adenomatous Glands*

Gland No. Weight of gland, gm.	1 216	2 130	3 274	4 152	5 238	6 115	7 223	8 83	9 124	10 111	Averages values for group
Per cent total N											
Amide N.....	10.01	9.97	9.71	10.28	10.25	9.50	9.96	9.86	10.00	9.79	9.93
Humin ".....	4.02	3.55	4.23	3.65	3.10	4.09	4.20	3.60	3.53	3.61	3.75
Arginine N.....	14.37	15.46	14.39	15.32	15.18	14.92	13.70	15.34	14.18	15.30	14.81
Cystine ".....	1.94	2.09	1.87	2.14	1.66	1.73	2.03	2.12	1.95	1.87	1.94
Histidine N.....	2.91	2.80	2.91	2.75	2.70	3.03	2.83	2.83	2.88	2.67	2.83
Lysine N.....	6.30	6.18	6.23	7.84	7.14	6.46	7.08	6.52	6.94	5.85	6.65
Filtrate amino N.....	55.72	55.94	55.85	54.93	55.80	56.31	55.54	55.96	56.76	57.47	56.02
" non-amino N.....	5.34	5.62	5.00	6.02	6.02	3.88	5.80	5.25	4.92	6.40	5.42
Per cent total protein											
Tryptophane.....	2.17	2.16	2.31	2.04	2.07		2.16	2.10	2.17	2.10	2.14
Tyrosine.....	3.45	3.33	3.11	3.17	3.33		3.33	3.19	3.08	3.12	3.23
" + tyrosine equivalent...	3.49	3.47	3.27	3.29	3.49		3.50	3.30	3.30	3.39	3.39
Thyroxine.....	0.0136	0.0382	0.055	0.066	0.073	0.083	0.113	0.116	0.194	0.24	0.099
Diiodotyrosine.....	0.10	0.13	0.33	0.26	0.37	0.33	0.38	0.21	0.46	0.53	0.31

these samples were obtained had all received Lugol's solution preoperatively.

In all the tables the tryptophane, tyrosine, thyroxine, and diiodotyrosine values are expressed as per cent of total protein and not as per cent of total nitrogen. The data included under "Tyrosine + tyrosine equivalent" are obtained by adding to the tyrosine values the amount of tyrosine which would be required to replace the diiodotyrosine and thyroxine in the thyroglobulin

TABLE IV

*Van Slyke Nitrogen Distribution of Thyroglobulin from Exophthalmic Glands*

Gland No. ....	1	2	3	4	6	7	9	Average
Weight of gland, gm. ....	74	93	66	156	95	67	325	values for group
Per cent total N								
Amide N. ....	9.93	9.67	10.00	9.99	9.72	9.95	9.40	9.81
Humin " . . . . .	3.93	4.40	4.44	4.35	3.80	4.23	3.42	4.08
Arginine N. ....	14.88	14.70	14.66	15.32	15.00	15.09	14.66	14.90
Cystine " . . . . .	2.02	1.87	1.95	1.91	2.10	2.04	2.10	1.99
Histidine N. ....	2.86	2.93	2.86	2.98	2.76	2.64	3.05	2.85
Lysine N. ....	7.06	6.77	6.99	6.52	6.69	6.68	6.43	6.73
Filtrate amino N. .	55.71	55.34	55.22	57.34	55.13	55.35	55.79	55.69
" non-								
amino N. ....	5.15	5.38	4.94	5.04	5.96	5.01	6.10	5.38
Per cent total pro-								
tein								
Tryptophane. ....		2.00	2.11	2.16	2.20	2.23	2.05	2.13
Tyrosine. ....		3.20	3.03	3.18	3.00	2.99	3.12	3.09
" + tyro-								
sine equivalent.		3.44	3.20	3.40	3.30	3.33	3.41	3.35
Thyroxine. ....	0.061	0.153	0.159	0.176	0.238	0.248	0.306	0.191
Diiodotyrosine. ....	0.273	0.50	0.33	0.46	0.61	0.70	0.55	0.489

molecule, assuming that a molecule of tyrosine replaces 1 molecule of diiodotyrosine or 1 molecule of thyroxine.

All of the Van Slyke nitrogen distribution data presented range between 99.4 and 101.98 per cent for recovery of total nitrogen.<sup>2</sup>

<sup>2</sup> The values given in the tables in some instances will total over 101.98 per cent as the cystine N present in the filtrate is included under cystine N as well as in the filtrate amino N.



## DISCUSSION

In comparing a group of analyses and their averages from one series with those obtained from another series it is very important that one have an idea of the experimental error involved in each method used for the analyses. The maximum variations in the analyses of each amino acid and nitrogen fraction of the normal thyroglobulin samples are included in Table V for this purpose.

If one studies the Van Slyke nitrogen distribution data of Tables I to IV, considering the maximum and minimum values found in each group and also the average values for the group given in Table V, it appears that the variations present for the different thyroglobulins, normal and pathological, are not significant because they lie within the experimental error of isolation and analysis.<sup>3</sup>

It will be noted that the tryptophane values for all analyses (Tables I to IV) vary from 2.00 to 2.31 per cent, being equally distributed among the four groups, and that the group averages are, respectively, 2.13, 2.15, 2.14, and 2.13 per cent tryptophane in thyroglobulin obtained from normal, colloid, adenomatous, and exophthalmic glands. This is very close agreement and indicates there is no variation in the tryptophane content of thyroglobulin from these various types of glands.

The tyrosine contents of the thyroglobulin from Tables I to IV show greater variation than can be accredited to experimental error. The variation in tyrosine content of samples of normal thyroglobulin with almost constant iodine content is very small (0.15 per cent). The average values (Table V) for each group showed more variation than errors in the method can account for, especially in the exophthalmic group where the variation was 0.2 per cent and the highest tyrosine content for any of these thyroglobulins from exophthalmic thyroids was less than the lowest value obtained in the normal series. This is undoubtedly a significant difference. However, on further study of the data it was noted that the tyrosine content varied somewhat inversely with the thyroxine and diiodotyrosine contents. Harington (4) has stated

<sup>3</sup> The experimental error present in these data is somewhat greater than that given by Cavett (2) for the method where variation in duplicate analyses on proteins was used.

TABLE V  
Average Values Given by Thyroglobulin from Normal and Pathological Glands

	Maximum variations found in normal group	Normal	Colloid	Variation from normal	Adenomatous	Variation from normal	Exophthalmic	Variation from normal
Per cent total N								
Amide N.....	2 91	9 33	9 82	+0.49	9 93	+0.60	9 81	+0.48
Humin ".....	1 47	3 93	3 69	-0.24	3 75	-0.18	4 08	+0.15
Arginine N.....	0 65	14 91	14 75	-0.16	14 81	-0.10	14 90	-0.01
Cystine ".....	0 60	1 91	2 02	+0.11	1 94	+0.03	1 99	+0.08
Histidine N.....	0 60	2 99	2 98	-0.01	2 83	-0.16	2 85	-0.14
Lysine N.....	1 95	6 84	6 45	-0.39	6 65	-0.19	6 73	-0.11
Filtrate amino N.....	1 41	56 77	56 49	-0.28	56 02	-0.75	55 69	-1.08
" non-amino N.....	1 87	4 90	4 80	-0.10	5 42	+0.52	5 38	+0.48
Per cent total protein								
Tryptophane.....	0 26	2 13	2 15	+0.02	2 14	+0.01	2 13	0.00
Tyrosine.....	0 15	3 29	3 38	+0.09	3 23	-0.06	3 09	-0.20
" + tyrosine equivalent.....		3 45	3 40	-0.05	3 39	-0.06	3 35	-0.10
Thyroxine.....		0 32	0 02	-0.30	0 10	-0.22	0 19	-0.13
Diiodotyrosine.....		0.277	0 05	-0.227	0 31	+0.217	0 49	+0.213

that it is probable that the iodine absorbed by the thyroid is first combined to form diiodotyrosine, some of which is converted to thyroxine. Also Alcock (5) has suggested that in the synthesis of protein, amino acids are disintegrated to simple groups which are built together, possibly by an enzyme system, to form an "urprotein," and from this heterogeneity of the amino acid products the specific rings and chains are added to the "urprotein" to produce the amino acids characteristic of the tissue protein.

To determine if this theory is supported by the above data it was assumed that each molecule of diiodotyrosine and thyroxine is located in the thyroglobulin molecule where a molecule of tyrosine has been. Thus in thyroglobulin from pathological glands, such as the colloid type, where no iodine medication had been used, one would expect the tyrosine content to be high for the diiodotyrosine and thyroxine content is low. This is true. Also, with thyroglobulin from a group like exophthalmic thyroids, where iodine therapy had been used, one would expect to find higher diiodotyrosine and thyroxine content and less tyrosine. This proved to be true.

An entry has been made in Tables I to V designated as "Tyrosine + tyrosine equivalent." These values were obtained by adding 23.31 per cent of the thyroxine content and 41.83 per cent of the diiodotyrosine content to the tyrosine content of the thyroglobulin. This gives the amount of tyrosine which would be present in the thyroglobulin molecule if a molecule of tyrosine were in the place of each diiodotyrosine and thyroxine molecule.

This theoretical assumption was almost true for the thyroglobulin from colloid thyroids where no iodine medication had been used. The average tyrosine value was 3.38 per cent, which is 0.09 greater than for normal thyroglobulin, while the "Tyrosine + tyrosine equivalent" was only 3.40 per cent. The other extreme was found in the thyroglobulin from exophthalmic glands, where iodine medication had been used. In these the average tyrosine value was 3.09 per cent, which is significantly lower than the average of 3.29 per cent for normal thyroglobulin, but the "Tyrosine + tyrosine equivalent" is 3.35 per cent, while that for normal thyroglobulin is 3.45 per cent, which is within experimental error.

It is noted in Table V that the "Tyrosine + tyrosine equivalent" gives much closer agreement between the normal and pathological

thyroglobulins than the tyrosine values do. It appears that the thyroglobulin molecule from the various types of pathological glands is not altered in its structure except that the pathological glands have difficulty in changing the tyrosine into diiodotyrosine and thyroxine. This is especially indicated in the colloid series (Table II) where no iodine medication was used. It is also noted in the adenomatous and exophthalmic series (Tables III and IV) where preoperatively 200 to 400 mg. of iodine were received daily. With the abundance of iodine, diiodotyrosine may be found, in some instances in much greater amounts than is present in normal thyroglobulin where no iodine medication was used, but in only one case does the thyroxine content approach that found in normal thyroglobulin.

#### SUMMARY

It appears from the data presented that the amino acid content of the thyroglobulin molecule, with the exception of thyroxine, diiodotyrosine, and tyrosine, is the same whether it be obtained from a normal, colloid, adenomatous, or exophthalmic gland.

The data seem to indicate that the tyrosine which is present at certain points in the thyroglobulin molecule is capable of conversion into diiodotyrosine or thyroxine. It appears that in the goitrous gland this formation, especially of the thyroxine, is interfered with.

The thyroglobulin from colloid glands where no iodine medication had been used is very deficient in the iodine-containing amino acids, and the tyrosine content is proportionately greater.

The adenomatous and exophthalmic glands, where iodine medication had been used, yielded thyroglobulin which contained varying amounts of the iodine-containing amino acids. The tyrosine content varied inversely with the amount of the iodine-containing amino acids.

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# THE DENATURATION OF PROTEINS BY SOUND WAVES OF AUDIBLE FREQUENCIES

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When egg albumin in aqueous solution at the isoelectric point is subjected to the action of intense sound waves of audible frequency, a coagulum is produced (1). A previous investigation (2) indicated that such sound-denatured<sup>1</sup> albumin is immunologically similar to denatured albumin prepared in other ways; *e.g.*, by heat in acid, alkaline, or neutral solution, by alcohol, or by acid or alkali.

Wu and Liu (3), making use of ultrasonic waves of much higher frequency than any employed by us, were able to coagulate albumin. However, when CO<sub>2</sub> or H<sub>2</sub>S was substituted for air in the solutions or when no gas at all was present, they observed no change with ultrasonic treatment. This contrasts with their observation that the reaction proceeds normally in the presence of pure O<sub>2</sub> or H<sub>2</sub>.

Although the results of our previous studies indicate that the end-product of sonic denaturation is probably the same as that produced by heating and therefore may be the result of a thermal type of activation, they leave unexplained the mechanism through which mechanical vibrations may energize the reaction. The peculiar relationship of the reaction toward certain gases reported by Wu and Liu has not clarified the mechanism for either range of frequency.

We have therefore undertaken a further study of the sonic denaturation of certain proteins, first, to compare the end-products of the reactions with those produced by other means with

<sup>1</sup> In this study the term denaturation is meant to imply the formation of a product of lower solubility than that of the native protein.

respect to chemical properties, and second, to obtain evidence as to the nature of the energy transfer underlying the reactions.

*Sonic Apparatus*—Two types of apparatus were used to produce the sonic vibrations. One was a modified electromagnetic oscillator of a type employed in submarine communication and depth finding. The vibrating element is a stainless steel diaphragm about 30 cm. in diameter and 18 mm. thick. Such a unit may produce approximately 175 watts of acoustic energy when operated at the resonant frequency of 1200 cycles per second. Use of the diaphragm as the bottom of a cylindrical metal container made possible the treatment of from 1 to 5 liters of solution in direct contact with the vibrating surface. This electromagnetic oscillator was used only in experiments designed to determine the effect of different frequencies on the reaction.

The apparatus more extensively employed was the modified magnetostriction oscillator described by Chambers and Gaines (4). It consists of a cold-drawn, unannealed nickel tube, vibrating in a strong magnetic field in resonance with a 2000 volt oscillating power circuit to which the tube imparts approximately its own natural frequency. The vibrator used in these experiments was of such length that a frequency of about 9000 cycles per second was produced.

Two types of accessory apparatus were used in the experiments. A heavy aluminum pressure vessel designed by one of us and described elsewhere (5) was used for treatment of solutions under pressure, while the glass vessel shown in Fig. 1 was used for exposures under a vacuum and in the presence of gases other than air.

*Materials*—Egg albumin was prepared from eggs less than 24 hours old according to a modified Hopkins method (6). The product was purified by four recrystallizations and was then well dialyzed and diluted to 1 per cent (Kjeldahl). Solutions of Merck's egg albumin were used for comparative purposes, but no qualitative difference in type of reaction was observed.

Native amorphous horse serum albumin was prepared by the technique of Anson and Mirsky (7). We did not crystallize the material since Svedberg and Sjögren (8) have shown that repeated recrystallization of serum albumin gives an increasingly heterogeneous mixture. The solution was diluted to 2 per cent and 100 ml. were acidified with 30 ml. of 0.1 N HCl. Such an acidified

solution will flocculate, if, after heating in a boiling water bath, the pH be raised again with 0.1 N NaOH to the isoelectric region.

Plastein was prepared by the technique of Wastenays and Borsook (9), by using pepsin in a concentrated peptic digest of egg albumin at pH 4. The solid plastein was then separated

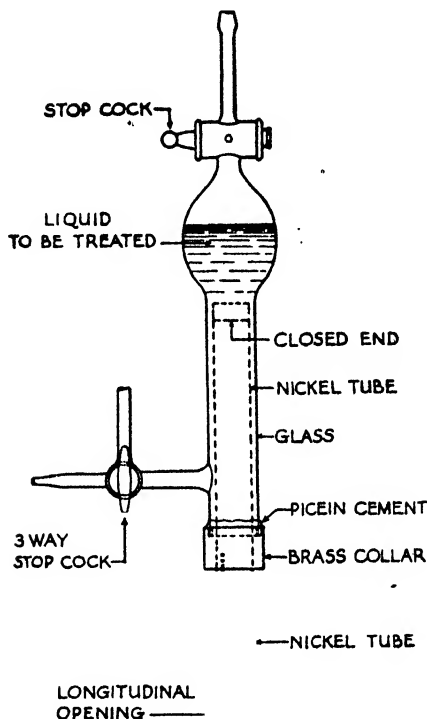


FIG. 1. Vacuum vessel

and well washed with water by centrifuging, and a solution of the material was prepared with 0.025 N HCl to a concentration of 1 per cent (Kjeldahl).

#### EXPERIMENTAL

When the 1 per cent egg albumin solution at the isoelectric point was vibrated in an open vessel, a white coagulum was



formed. In a 25 ml. sample approximately 20 per cent of the protein was so denatured in 4 minutes, although the amount varied from time to time owing to variations in acoustic output of the oscillator. This coagulum is soluble in dilute acid or alkali only to the extent of about 0.03 per cent (2). No part of the denaturation may be attributed to mass temperature rise in the solution, since adequate water cooling was provided to prevent an increase in temperature of more than 2° or 3°.

Prolonged treatment continued the production of coagulum but at the same time dispersed it into an opalescent sol which could not be separated by centrifuging at ordinary speeds (100 × gravity). In our previous study (2) no difference in immunological specificity between the products of moderately prolonged and short treatments was found; each gave reactions similar to those obtained with heat-denatured egg albumin.

Very prolonged sonic treatment (6 hours) apparently results in a further change in the coagulum (2), but we have not concerned ourselves with the nature of that end-product in this communication.

An investigation of the influence of the hydrogen ion concentration on sonic denaturation seems to corroborate the immunological finding that we are dealing with a thermal type of denaturation in the case of egg albumin. No coagulum was formed by 4 minutes vibration of the solution adjusted to pH 7.2 by addition of 0.15 N NaOH. (For some reason, unexplained as yet, the pH value fell to 6.7.) However, a shift in pH to the isoelectric point by adjustment with 0.025 N HCl subsequent to sonic treatment resulted in flocculation of 13 per cent of the original protein. Furthermore, the flocculated product was found to dissolve readily in excess of either acid or alkali. All of these peculiarities with respect to the conditions of pH parallel exactly those encountered when heat is the denaturing agent. Similarly, an acid solution of plastein was coagulated by sonic vibration under the pH conditions required for its coagulation by heat.

However, no such parallelism was observed in the case of horse serum albumin. Heat caused coagulation of a solution of this protein at pH 6, but no change was observed even by prolonged sonic treatment at this pH. Horse serum albumin vibrated at lower pH did not flocculate upon subsequent addition of 0.1 N

NaOH, although flocculation proceeded normally in a control portion when heating was substituted for the mechanical vibration. The chemical behavior of horse serum albumin differs from that of egg albumin in certain other respects. For example, the reversal of the denaturation of horse serum albumin is easily accomplished, but the reversal of denatured egg albumin has not been possible up to the present time (7, 10). Whether there is a relation between those proteins the heat denaturation of which is not reversible and those which are susceptible to sonic denaturation remains to be determined.

Although there is no reason thus far to doubt that the end-products of sonic and thermal denaturation are the same, when and if such products are formed, nevertheless the experiments give evidence that the mechanism of sonic denaturation does not parallel that of heat denaturation, as the studies on egg albumin alone seemed to indicate. Furthermore, the failure of horse serum albumin to be denatured indicates that the denaturations are not caused by possible momentary, localized temperature increases in the liquid resulting from adiabatic compressions, or from cavitation collapse. We have, therefore, undertaken a study designed to shed light on the mechanism through which sound waves energize the reaction.

When a liquid is exposed to the action of a strongly oscillating diaphragm or piston cavitation occurs; that is, evanescent vacuoles appear in the body of the fluid and at the surface of the vibrating element. The formation of these low pressure spaces has been discussed in detail by Gaines (11), and others, and practically all biological and chemical effects of intense sound heretofore described have been attributed either to the formation or collapse of the cavities. Since cavitation is the most obvious visible manifestation of sonic action on liquids, experiments were carried out to determine what relation exists between the denaturation reaction and the formation of the characteristic, vigorously active, gaseous or vapor bullæ. At the acoustic intensities available in the present study cavitation is produced in water with or without the presence of a dissolved gas, and only when an external pressure of approximately 6 atmospheres is applied to the liquid is cavitation completely inhibited.

On the other hand, the ultrasound source used by Wu and Liu

was reported by them to cause "gas bubbles" (cavitation) at atmospheric pressure in the presence of some gases (air,  $O_2$ , and  $H_2$ ) but not in the presence of certain others ( $CO_2$  and  $H_2S$ ). Furthermore, no cavitation was caused in gas-free water (3). Wu and Liu found that egg albumin is coagulated by ultrasonic treatment under those conditions which allowed cavitation, and that no change is caused when the solutions fail to cavitate. From this observation they conclude that coagulation is brought about by condensation at the surfaces of the "gas bubbles," a reaction which can also be caused by vigorous manual shaking of the albumin solution in air.

A surface condensation mechanism, however, would not adequately explain the production in acid solution of a denatured product that is insoluble only at the isoelectric point. While our studies in the audible frequency range demonstrate that cavitation is essential to the reaction, further evidence made available by the possibility of producing cavitation in gas-free water and in water saturated with any type of gas, together with certain other observations recorded below, render improbable the conclusion of Wu and Liu regarding condensation at gas surfaces. It is true that coagulation of isoelectric egg albumin was not produced even by prolonged vibrations when cavitation in an air-saturated solution was completely inhibited by imposition of a hydrostatic pressure of 100 pounds per square inch. It is therefore evident that the denaturation occurs as a result of, or simultaneously with cavitation. However, one may not conclude from this fact that the sonic coagulation process is a surface phenomenon. Coagulation by shaking the solution with air may be inhibited by the addition of a surface-active substance such as saponin, but addition of saponin to solutions of egg albumin does not prevent coagulation by sound at normal pressure to any measurable degree.

Furthermore, sonic denaturation is not produced under all circumstances even when adequate cavitation is present. Although a gas-free solution of egg albumin literally boils during vibration under a vacuum, no coagulation results upon such treatment at the isoelectric point. Nevertheless, it seems definitely established that cavitation of the protein solution must occur in order that denaturation may take place.

In our experiments within the audible frequency range a specificity of the reaction toward certain gases was observed even though the acoustic intensity was such that cavitation was vigorous with all types of gases. Thus with air, oxygen, or carbon dioxide as the dissolved gas, coagulation of egg albumin solution treated at the isoelectric point reaches a maximum rate when pressure conditions allow ample cavitation; *i.e.*, at atmospheric pressure. On the other hand, with nitrogen or hydrogen coagulation was very nearly, if not completely, prevented.<sup>2</sup> The reaction was certainly very much more rapid when cavitation was ample in the presence of  $O_2$  or  $CO_2$  than was the case either with diminished cavitation, or with vigorous cavitation in the absence of dissolved gas or in the presence of hydrogen or nitrogen. The negative results with these two gases constitute evidence that sonic coagulation is not a surface condensation due to shaking.

We are dealing, then, with a reaction which requires, first, a sufficiently dense acoustic field to produce vigorous cavitation, and second, the presence of certain gases, *viz.*  $O_2$  or  $CO_2$ . The implication seems clear that the denaturation is not a result of direct absorption of sound energy by the protein molecule, but is rather the result of an energy transfer through intermediary activated gas molecules. Furthermore, one may safely assume that the sonic activation of  $O_2$  (demonstrated by Flosdorf, Chambers, and Malisoff (12)) and  $CO_2$  in this sense takes place only under conditions favoring cavitation.

Such an energy transfer takes no account of chemical interactions between the activated gases or their products and the albumin molecule. This possibility should not be ignored. It is, however, difficult to reconcile the observation that both  $CO_2$  and  $O_2$  plus acoustic energy result in identical denatured end-products with any theory involving chemical combination. In the case of  $O_2$  it has been shown by Flosdorf, Chambers, and Malisoff (12) that hydrogen peroxide is formed as a result of sonic activation in aqueous solution. The possibility of dena-

<sup>2</sup> It was difficult to determine whether or not there was complete absence of change, since there was always a trace of coagulated material present in the solutions as a result of the necessary preliminary manipulations. The detection of minute amounts of sonically produced coagulum was therefore impossible.

turation by peroxide was therefore checked by hand shaking and by sonic treatment of albumin solutions with  $\text{H}_2\text{O}_2$  in concentrations of the order known to be present after 4 minutes vibration. No increased denaturation resulted. It appears then that the gas functions only as a carrier of energy to the protein molecule. That the transfer can be effected by certain gases and not by others under the same conditions may be correlated with the observations of Knudsen and Kneser (13) and of Kneser (14) that  $\text{CO}_2$  and  $\text{O}_2$  absorb far more acoustic energy at audible frequencies than does  $\text{N}_2$  or  $\text{H}_2$ . We realize that a hypothesis based on similarities between measurements made on materials in the gaseous state in the one case, and in a state of solution in the other, are precarious and at best can leave the specificity of the gases only inadequately explained. We are, nevertheless, impressed by the fact that of the four gases investigated the two gases showing maximum absorption and the two showing reaction specificity in our experiments are the same.

The wide differences in specificity toward  $\text{N}_2$  and  $\text{CO}_2$  represented by our findings at audible frequencies and by those of Wu and Liu at ultrasonic frequencies suggest that extensive investigation over a wide range of frequencies will be necessary to account for the discrepancies.

We wish to express our appreciation to Dr. Stuart Mudd for his helpful criticisms in this work.

#### SUMMARY

Egg albumin and plastein are denatured by intense sonic vibration. The solubility of the products under various conditions of pH is the same as that of the heat-denatured products.

Horse serum albumin is not denatured by sonic vibration. This fact suggests that the mechanism of the sonic reaction may differ from simple thermal activation.

Denaturation of egg albumin occurs only at acoustic intensities sufficient to promote vigorous cavitation of the solution. However, even at these intensities there is no denaturation when cavitation is suppressed by pressure or when cavitation is ample in the absence of dissolved gas.

The reaction proceeds actively when air,  $\text{CO}_2$ , or  $\text{O}_2$  is present,

but not in an atmosphere of either  $N_2$  or  $H_2$ , or in gas-free solution under a vacuum, even when the solution is vigorously cavitated.

A possible mechanism of sonic denaturation is discussed. The hypothesis requires direct transfer of energy from activated gas to protein molecules without chemical interaction.

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## THE CHEMISTRY OF MOLD TISSUE

### XI. ISOLATION OF LEUCINE AND ISOLEUCINE FROM *ASPERGILLUS SYDOWI*\*

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In view of the rather high percentage of nitrogen in molds it is surprising to note the scarcity of reports concerning their amino acid content. Few amino acids have been reported, and still fewer have been isolated and characterized by analysis and by the formation and examination of suitable derivatives. Leucine, both free (1) and combined (2), has been reported in *Aspergillus niger*, but no report of the presence of isoleucine in molds has been found.

By continuous extraction of the dry, defatted mycelium of *Aspergillus sydowi* with acetone for several weeks we have obtained a mixture of amino acids from which leucine and isoleucine have been isolated in considerable quantities and identified. It would thus appear that at least these two amino acids occur free in the mycelium, or that they are very loosely combined and are split off during drying and extraction of the material.

In the separation and purification of a mixture of amino acids whose properties are so nearly alike as those of leucine and isoleucine considerable losses are inevitable. For this reason, the quantities isolated represent the minimum amounts present; the actual amounts present are undoubtedly much greater. Of the defatted mold, 0.39 per cent was isolated as leucine, and 0.87 per cent as isoleucine. However, if all the amino nitrogen of the extract is assumed to be due to leucine and isoleucine, these two amino acids were obtained to the extent of 2.3 per cent of the defatted mold.

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## EXPERIMENTAL

*Extraction of Mycelium*—The mold was grown on glucose-inorganic salts medium in large sterilizer incubators as previously described (3). 900 gm. of dried, ground, defatted<sup>1</sup> mycelium were placed in a large Soxhlet type extractor, and extracted continuously for several weeks with acetone. Large amounts of white, crystalline material separated out in the boiling flask beginning about the 1st day. From time to time as the material accumulated, it was filtered off from the acetone, and the extraction continued with fresh acetone. In a pilot run, with 110 gm. of defatted mold, 11.3 gm. of material were obtained after 18 days. Further extraction for 6 days yielded only 0.4 gm. of material, but the mold still contained amino acids, as shown by the ninhydrin reaction. With the large extractor, which did not siphon as often as the smaller one, 69 gm. were obtained after 3 weeks. Further extraction yielded small quantities of material.

*Fractionation of Extract*—The 69 gm. of solids were warmed with water and filtered from insoluble matter amounting to 5 gm. The water solution contained 3.00 gm. of N (Kjeldahl) and 2.19 gm. of  $\alpha$ -amino N (Van Slyke). To this solution was added the water solution of the extract from the pilot run mentioned above. The total water solution then contained 3.36 gm. of N and 2.46 gm. of  $\alpha$ -amino N.

To remove purines and similar compounds that may have been present, the solution was concentrated under reduced pressure to 400 cc., and was then treated with a solution of mercuric chloride. A small amount of light yellow precipitate formed and was filtered off. About 6 per cent of the total nitrogen was precipitated by this treatment. In order to separate the amino acids from the large amounts of mannitol<sup>2</sup> and other non-nitrogenous compounds, the solution was now treated with sodium

<sup>1</sup> The mold was defatted by extraction with alcohol-ether (1:1) and was found to contain 12 per cent crude lipids.

<sup>2</sup> The presence of mannitol was proved by evaporating to dryness some of the original water solution of the extract from a separate sample of the same batch of mold, decolorizing with norit, and recrystallizing twice from alcohol. Typical mannitol crystals, melting at 165°, were obtained. The acetate was prepared and was found to melt at 120°. The melting points of mannitol and its acetate are 166° and 119° respectively.

carbonate and mercuric acetate (Neuberg's reagent), and alcohol was added to a concentration of 25 per cent. The resulting precipitate was filtered off and decomposed with  $\text{H}_2\text{S}$ .

About 29 per cent (980 mg.) of the total nitrogen was not precipitated by Neuberg's reagent. None of this nitrogen reacted by the Van Slyke method. After concentration of the filtrate, a second treatment with sodium carbonate and mercuric acetate failed to precipitate any of this nitrogen. The mercury was removed from this filtrate, and to the resulting filtrate was added an equal volume of alcohol. No nitrogenous matter was precipitated by this procedure. As yet no nitrogen compounds have been isolated from the Neuberg filtrate.

*Mercuric Chloride Precipitate*—The precipitate obtained with mercuric chloride was decomposed with  $\text{H}_2\text{S}$ , filtered, and after removal of the excess  $\text{H}_2\text{S}$ , the filtrate was treated with ammoniacal silver nitrate. In this way the material was divided into two fractions, neither of which gave a positive ninhydrin test. No compounds have been identified in either fraction.

The filtrate from the decomposed Neuberg precipitate was evaporated to about 350 cc. under reduced pressure at  $40^\circ$ . This solution contained 2.30 gm. of N (Kjeldahl) and 2.20 gm. of N by the Van Slyke method.

*Isolation of Leucine*—The solution was boiled up with an excess of freshly precipitated copper hydroxide, and filtered while hot. The residue was entirely inorganic. The filtrate was evaporated to dryness, triturated with cold water, and washed with cold water until the washings were nearly colorless. About 1200 cc. of water were used. The light blue residue was dried *in vacuo*.

$(\text{C}_6\text{H}_{12}\text{O}_2\text{N})_2\text{Cu}$ . Calculated, N 8.66; found, N 8.66

The copper salt weighed 4.84 gm., which corresponds to 3.88 gm. of leucine. This represents 0.39 per cent of the defatted mold.

The copper salt was decomposed with  $\text{H}_2\text{S}$  and the filtrate concentrated at  $40^\circ$  under reduced pressure until crystallization began. Alcohol was added, and the crystals were filtered off and dried; 2.6 gm. were obtained. In water solution  $[\alpha]_D = -10.3^\circ$  (calculated on the basis of the weight of the solution taken, not its volume).

$\text{C}_6\text{H}_{11}\text{O}_2\text{N}$ . Calculated, N 10.7; found, N 10.6

The molecular weight by Linderstrøm-Lang titration with HCl (4) was 133, and by alcoholic NaOH titration to the thymolphthalein end-point was 130. Leucine has a molecular weight of 131. The copper salt was prepared and dried *in vacuo*.

$(C_6H_{13}O_2N)_2Cu$ . Calculated, Cu 19.64; found, Cu 19.61

0.1486 gm. was oxidized with 1 mole of chloramine T, and the aldehyde so formed was distilled into a solution of *p*-nitrophenylhydrazine in dilute acetic acid. The resulting hydrazone was recrystallized twice from dilute alcohol. Melting point 108°. The *p*-nitrophenylhydrazone of isovaleraldehyde melts at 109° (5).

*Isolation of Isoleucine*—The dark blue filtrate from leucine copper salt was evaporated to dryness and thoroughly dried *in vacuo*. The salts were finely pulverized, triturated with absolute methyl alcohol, and filtered by suction. This treatment was repeated until the filtrate was only pale blue. About 2 liters of methyl alcohol were used. The residue weighed 3.7 gm.

The methyl alcohol was distilled, the residue was dissolved in water, decomposed with  $H_2S$ , filtered, concentrated at 40° under reduced pressure until crystallization began, treated with alcohol, and placed in the ice box until crystallization was complete. The crystals weighed 3.3 gm. By concentrating the filtrate and adding acetone 4.6 gm. more were obtained. In water solution  $[\alpha]_D = +10^\circ$ . Values in the literature range from +9.6° to 11.3° (6). Abderhalden and Zeisset (7) have recently reported 10.8°.

$C_6H_{13}O_2N$ . Calculated, N 10.7; found, N 10.6

The molecular weight by Linderstrøm-Lang titration was 128 (theoretical, 131). 0.1414 gm. was oxidized with chloramine T, and the *p*-nitrophenylhydrazone of the aldehyde was prepared. After recrystallization from dilute alcohol this derivative melted at 112–113°. Neuberg and Peterson (8) have reported 112–113° for active methylethylacetaldehyde-*p*-nitrophenylhydrazone. The hydrazone separated first as an oil, but crystallized after standing in the ice box for some time.

The phenyl isocyanate derivative was prepared in the usual way from 0.149 gm. of the amino acid by the action of phenyl isocyanate and NaOH at 0°. After crystallization from water it

melted at 120°. Abderhalden and Zeisset (7) found that the *d*-isoleucine phenyl isocyanate derivative melted at 121°.

The *p*-toluenesulfonate of 0.26 gm. was prepared according to the procedure used by Fischer and Lipschitz (9). It was recrystallized from water. Beautiful needles were obtained which melted at 122.5° (124° corrected). The melting point was not changed by further recrystallization.

$C_{13}H_{19}NO_4S$ . Calculated, N 4.91; found, N 4.83

We have not found any reference to this compound in the literature.

*Methyl Alcohol-Insoluble Copper Salt*—When treated with water, the methyl alcohol-insoluble residue (3.7 gm.) did not all dissolve. A green, amorphous precipitate (probably copper hydroxide or carbonate) weighing 0.66 gm. was filtered off and discarded. The copper was removed from the filtrate with  $H_2S$ , the filtrate was concentrated under reduced pressure, and the amino acid precipitated with acetone; 0.8 gm. was obtained. This material was recrystallized from alcohol.

$C_6H_{13}O_2N$ . Calculated, N 10.7; found, N 10.6

The *p*-toluenesulfonate melted at 120–122° and contained 4.7 per cent nitrogen. The *p*-nitrophenylhydrazone formed from the aldehyde produced by oxidizing the amino acid with chloramine T melted at 109–110° and showed the same crystalline form and behavior as the *p*-nitrophenylhydrazone obtained from isoleucine. A mixture of the two hydrazones melted at 110°. These facts indicate that the methyl alcohol-insoluble copper salts consisted of the copper salt of isoleucine occluded by copper hydroxide or carbonate. Thus, a total of 8.7 gm. of isoleucine was isolated from the water-soluble copper salts. This corresponds to 0.87 per cent of the defatted mold.

#### SUMMARY

Leucine and isoleucine have been isolated in considerable quantities by extracting the dried, defatted mycelium of *Aspergillus sydowi* with acetone. From this method of extraction it was concluded that these amino acids must be present either free in the mycelium, or held in rather loose combination. The amino

acids were characterized by analysis and by the preparation and examination of two or more derivatives. The *p*-toluenesulfonate of isoleucine has been prepared apparently for the first time.

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## CANINE CYSTINURIA

### II. ANALYSIS OF CYSTINE CALCULI AND SULFUR DISTRIBUTION IN THE URINE

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In 1810 Wollaston (1) discovered a previously unknown variety of human urinary calculi, consisting of a new chemical compound to which the name cystine was assigned. The first cystine stone obtained from a dog<sup>1</sup> was described a few years later by Lassaigne (2), and other examples of such stones have since been reported (3). It may be concluded, therefore, that cystinuria occurs in dogs; but, as Garrod (4) stated in 1923, the diagnosis has hitherto been made only after the death of the animal. It is interesting to note, however, that "A Rare Form of Urolithiasis Associated with Clonic Spasms of Muscles (Chorea) of the Posterior Limbs of the Dog" was reported in 1921 in a male Dachshund (5). The author states that, "It was found that the calculi were entirely composed of what is termed cystin;" the urine was not investigated.

We have recently described a case of cystinuria in a male Irish terrier and have given a detailed presentation of the clinical aspects of this case (6). We are reporting in the present paper on the chemical composition of the cystine calculi and on the sulfur partition in the urine of this cystinuric dog.

A large calculus<sup>2</sup> weighing 6.5 gm. and numerous small calculi were removed by operation from the bladder and the urethra of the dog (6). It can be seen from Table I that the calculi consisted

<sup>1</sup> The question whether the dog calculus analyzed by Lassaigne was really composed of cystine will be discussed in a subsequent publication.

<sup>2</sup> For a colored photograph of this calculus cf. (6).

mainly of cystine (94 and 88 per cent). A total sulfur determination (Pregl) on the large stone checked within experimental error with the colorimetric determination for cystine, indicating the probable absence of other sulfur compounds. Cystine crystals

TABLE I  
*Chemical Analysis of Cystine Calculi*

	Large stone	Small stones		Large stone	Small stones
	per cent	per cent		per cent	per cent
Moisture.....	0		Total S.....	25 5	
Ash.....	1		Cystine S...	25 2	
Phosphorus.....	0 07	0 19	Undetermined S..	0 3	
Cystine (photometric)			Total N.....	11 57	11 63
(7).....	94	88	Cystine N.....	10 95	10 25
Cystine (Sullivan) ...		88	Allantoin N	0 09	0 05
Allantoin (8).....	0 26	0 14	Undetermined N...	0 53	1 33

TABLE II  
*Sulfur Partition and Cystine in a Casual Specimen of Urine from Cystinuric Dog*

Sulfur					Cystine	
Total	Total SO <sub>4</sub>	Neutral			Folin photometric method	Lugg-Sullivan method
		Total	Cystine	Undetermined		
gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.
0 52	0 24	0 28	0 09	0 19	0 34	0 33
Per cent of total S						
	46	54	17	37		
Per cent of neutral S						
			31	69		

prepared from the large stone and twice recrystallized showed an optical rotation of  $[\alpha]_D^{20} = -211^\circ$  in 0.2 N HCl, which rotation did not increase on further recrystallization.

The determination of total nitrogen (micro-Kjeldahl) indicated the presence of other nitrogen-containing substances besides

cystine. Urea and uric acid were absent, but the furfural test for allantoin was positive. A quantitative estimation of allantoin by the method of Allen and Cerecedo (8) yielded small amounts of this substance (0.26 and 0.14 per cent, respectively). There remained, unidentified, a certain amount of nitrogen (0.53 and 1.33 per cent N, respectively).

Several casual specimens of urine from the dog were analyzed for various sulfur fractions and for cystine by the methods used in previous publications (7, 9, 10). It can be seen from Table II that the sulfur partition in this sample was typical for cystinuria, the total sulfates amounting to less than 50 per cent of the total S. The determination of cystine yielded the same result (0.33 gm. per liter) both with the photometric Folin method and with the Lugg-Sullivan method. Only 30 per cent of the neutral sulfur was accounted for by cystine. 70 per cent of the neutral S, *i.e.* as much as 37 per cent of the total S excreted in the urine, remained unidentified.

Cystinuria is not a characteristic of the Irish terrier, because twenty-five animals of this breed showed no evidence of cystine excretion. However, we have recently observed a male Irish terrier, whose urine gave a strongly positive cyanide-nitroprusside test, but failed to give Sullivan's reaction. Nevertheless, the urine contained appreciable amounts of cystine, since a positive Sullivan's reaction was obtained when the cuprous mercaptide method (11) was applied. This finding indicated that the dog had cystinuria. It is interesting to note that an examination of the pedigree showed that the dog was the son of a male litter mate of the original cystinuric dog. The study of this second dog is being continued.

The original cystinuric dog was mated with two half-sisters, and with an unrelated female of the same breed. Three litters, a total of fifteen pups, were obtained. None of these pups has cystinuria, but it may be expected that interbreeding will re-establish the disease.

#### SUMMARY

1. Cystine calculi from a dog contained a small amount of allantoin and some other unidentified nitrogen.

2. A casual specimen of urine showed a typical cystinuric



distribution of sulfur. However, cystine accounted for only 30 per cent of the neutral sulfur.

3. Some evidence regarding the hereditary nature of canine cystinuria is presented.

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## THE INFLUENCE OF MILK CONSTITUENTS ON THE EFFECTIVENESS OF VITAMIN D

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PLATES 2 AND 3 ·

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The peculiar and marked clinical efficacy of irradiated milk was recognized by Scheer (1) as early as 1928. Later reports by Hess and Lewis (2) emphasized the lack of correlation between "potency" as numerically expressed in terms of "vitamin D rat units" and the clinical effectiveness of cod liver oil, viosterol, milk from cows fed irradiated yeast, and irradiated milk. Numerous comparative studies have since been reported, wherein the "unit" potency of different antirachitic agents is correlated with the clinical results. The numerical ratio of clinical effectiveness of irradiated milk to other antirachitic agents has not always been confirmed by these later data. The discrepancies, however, are predicated upon the implied assumption that the "rat unit" is an infallible criterion of the physiological response due to vitamin D irrespective of the manner in which it may exist in dietary or pharmaceutical substances. If it is assumed that the assay results have been uniformly interpreted, an explanation for the lack of parallelism between "unit potency" and clinical effectiveness of various antirachitic agents is not yet available.

Various theories have been offered to explain the discrepancy between the empirical laboratory criterion and the clinical results obtained with irradiated milk. Watson (3) attributes the efficacy of irradiated milk to a "vital" factor which is not measurable by the established procedures. It has been suggested also that the antirachitic factors from different sources are not identical (4), the vitamin D of animal origin being more efficient than that of

plant origin (5). A recent report states that the rat unit of natural vitamin D is about 100 times more potent in protecting chickens and children from rickets than the rat unit of irradiated ergosterol (6). The inherent calcium and phosphorus content of milk has frequently been suggested as the reason for the particular merits of irradiated milk; likewise, the inherent cholesterol content of milk may be a contributing factor. A further possible explanation is suggested by the data published in a previous paper from these Laboratories (7), wherein it was shown that the provitamin in milk is not entirely confined to the butter fat, and that the lipid matter prosthetically bound with the lactalbumin and possibly other milk proteins could be activated to a measurable degree.

In a recent paper Lewis (8) reports the striking observation that pure vitamin D dissolved in a vehicle miscible with water is more effective clinically when added to milk than when the same amount of the pure vitamin is administered in an oil vehicle. In view of the suggested explanations of the superior clinical effectiveness of irradiated milk, certain experiments have been carried out with consideration to those factors which might possibly influence the effectiveness of vitamin D.

#### EXPERIMENTAL

Since the Ca:P ratio of the diet affects the vitamin D response in experimental animals, it was first necessary to determine variations in the line test, which might be attributed to changes in the Ca:P ratio caused by different levels of milk supplementing the Steenbock rachitogenic Diet 2965 (9). The results from these comparative tests are summarized in Table I. The Ca:P ratio as ingested was computed from the daily intake of the basal ration during the 10 day supplemental feeding period and the amount of calcium and phosphorus carried by the test substance. In all comparative tests involving the use of irradiated milk, the conditions of irradiation were standardized; suitable controls were likewise established for all other comparable tests.

Since the numerical unit designation is not appropriate for many research purposes, the average degree of calcification (numerical average from all rats in the same group receiving the same test substance at the same level), as revealed by the line test determined according to standard procedures, has been used as the

criterion for determining differences in the response of the test animals. In order that the significance of the tabulated records may be more clearly understood, typical photomicrographs of the degrees of calcification, employed as reference standards in these Laboratories for many years, are reproduced in Figs. 1 to 9.

The data presented in Table I show that increased healing response is obtained with increasing amounts of non-irradiated milk, and that this response is greatly accentuated if the milk is irradiated. The adjustment of the Ca:P ratio of the basal ration to that which prevails when different levels of milk are fed is ineffective in promoting healing in the absence of milk. However, when the Ca:P ratio is reduced to 2.70 (equivalent to that which is ingested with 30 ml. of milk per day as a supplement to the basal ration) and as low as 4 ml. of non-irradiated milk is supplied daily, a marked healing response is noted, the healing being further accentuated if the milk is irradiated. Reduction in the calcium and phosphorus content of cow's milk treated by the base exchange method (10) does not significantly affect the healing response from the irradiated and non-irradiated milks so treated, as compared with parallel samples of natural milk.

The failure of composited samples of irradiated human milk to induce any significant degree of healing was a surprising result. The data as a whole seem to indicate that cow's milk possesses certain characteristics, other than the Ca:P ratio, which tend to accentuate the healing of rickets; this property, inherent in the non-irradiated product, is intensified several fold by irradiation.

In 1931 (11) attention was directed to a peculiar physico-chemical relationship between the provitamin and milk constituents, and in 1934 (12) data were presented showing that there was no parallelism between the antirachitic potency of irradiated milk and its fat content. These and other observations have directed attention to constituents of milk other than fat, calcium, and phosphorus, as a possible explanation for the antirachitic properties of the irradiated product.

Numerous conditions exist in natural products wherein the provitamin and the vitamin may be associated with substances dispersed in an aqueous medium. Pure vitamin D in water-miscible propylene glycol permits a broader field of investigation than is possible when the vitamin is confined to an oil vehicle. A

TABLE I  
Influence of the Ca:P Ratio, Milk, and Irradiated Milk on Healing of Rickets

Test substance and Ration 2965				Amount fed per day	Ca:P	Average degree of calcification
				ml.		+
Ration 2965 only.....				<i>Ad libitum</i>	4.51	0.0
Ca and P adjusted to equivalent of 2 ml. milk daily.....				" "	4.20	0.0
" "	" "	" "	2.7	" "	4.15	0.0
" "	" "	" "	4	" "	3.90	0.0
" "	" "	" "	10	" "	3.53	0.0
" "	" "	" "	30	" "	2.70	0.5
" "	" "	" "	30	+ non-irradiated milk.....	2.65	2.2
" "	" "	" "	30	+ irradiated milk.....	2.65	3.9
Non-irradiated milk.....				2	4.20	0.0
Irradiated milk.....				2	4.20	1.0
Non-irradiated milk.....				2.7	4.15	0.0
Irradiated milk.....				2.7	4.15	1.6
Non-irradiated milk.....				4	3.90	0.2
Irradiated milk.....				4	3.90	2.1
Non-irradiated milk.....				10	3.53	1.0
Irradiated milk.....				10	3.53	3.5
Non-irradiated milk.....				30	2.70	3.1

Irradiated milk (Ca:P 1.41) .....	4	3.90	2.4
Non-irradiated soft curd milk (0.098% Ca, 0.073% P, Ca:P 1.34) .....	4	3.95	0.6
Irradiated soft curd milk (0.098% " " 1.34) .....	4	3.95	2.4
Non-irradiated soft curd milk (0.035% " " 0.58) .....	4	4.01	0.6
Irradiated soft curd milk (0.035% " " 0.58) .....	4	4.01	2.5
Irradiated human milk (4.10% fat) .....	4	4.43	0.5
" " (4.30% " ) .....	4	4.43	0.6
" " (4.30% " ) irradiated 3 times .....	4	4.43	0.6
" " (4.30% " ) diluted to 6.8% solids and irradiated 3 times .....	4	4.43	0.9

0.05 per cent solution of pure vitamin D in propylene glycol<sup>1</sup> and solutions of highly dispersed lactalbumins have been particular objects of study. Two types of lactalbumin were prepared from milk by precipitation at the isoelectric point, following removal of the casein. Repeated washing with hot water and reflocculation at the isoelectric point yielded a substantially pure protein with prosthetically bound lipid material (Lactalbumin 7-HAA) of the following composition.

	<i>per cent</i>
Nitrogen.....	14.15
Cystine.....	2.68
Reducing carbohydrates (lactose).....	None
Lipid matter (sterols, phospholipids, etc.) .....	6.83
Cholesterol .....	0.30
Sulfur .....	1.75
Phosphorus .....	0.14
Ash.....	0.50
Calcium.....	None
Magnesium.....	"
Chlorine.....	"

The lipid matter was removed by extracting the freshly prepared product with suitable solvents, thereby obtaining a further purified lactalbumin devoid of this material (Lactalbumin 7-HAAX). These lactalbumins were suspended in water at 0.02 per cent concentration, thoroughly dispersed with weak sodium hydroxide, and the pH then adjusted to a value slightly on the acid side of neutrality. Varying amounts of the vitamin D solution were added to these highly dispersed lactalbumins. Following agitation and holding at a low temperature (between about 1–10°), the solutions were fed to properly prepared rachitic rats at the rate of 1.2 mg. of lactalbumin per day (6 ml. of the colloidal solution) for a period of 10 days. The same amounts of the vitamin D in water were also fed.

Other solutions of highly dispersed lactalbumin and vitamin D were similarly prepared and the protein precipitated at its isoelectric point at a temperature of 50°. The precipitate was removed by filtration, washed several times with water at the isoelectric point, resuspended and dispersed in weak sodium hydrox-

<sup>1</sup> The propylene glycol solution of vitamin D was obtained from the Winthrop Chemical Company, Inc.

ide, and the pH adjusted to a value slightly below the neutral point. These redispersed lactalbumins were fed at the same levels as in the previous series (1.2 mg. of lactalbumin per day). The results are recorded in Table II, from which it will be noted that

TABLE II

*Antirachitic Response from Unit Amounts of Pure Vitamin D As Influenced by Dispersed Lactalbumin Solutions*

Solution carrying vitamin D	Amount of vitamin D fed per day	Average degree of calcification
	micrograms	+
Water.....	0.06	0.0
Lactalbumin containing lipids .....	0.06	0.5
"    lipid-free.....	0.06	1.5
"    containing lipids, redispersed.. ..	0.06	0.3
"    lipid-free, redispersed .....	0.06	0.5
Water.....	0.15	0.0
Lactalbumin containing lipids .....	0.15	1.6
"    lipid-free.....	0.15	1.8
"    containing lipids, redispersed .....	0.15	No data
"    lipid-free, redispersed .....	0.15	1.5
Water .....	0.37	1.6
Lactalbumin containing lipids .....	0.37	2.1
"    lipid-free.....	0.37	3.6
"    containing lipids, redispersed .. ..	0.37	2.0
"    lipid-free, redispersed .....	0.37	2.9
Water .....	0.90	2.1
Lactalbumin containing lipids .....	0.90	2.5
"    lipid-free.....	0.90	No data
"    containing lipids, redispersed .....	0.90	2.8
"    lipid-free, redispersed .. ..	0.90	3.4
Water.....	1.50	3.4
Lactalbumin containing lipids .....	1.50	No data
"    lipid-free.....	1.50	" "
"    containing lipids, redispersed .....	1.50	3.1
"    lipid-free, redispersed .....	1.50	2.9

the healing response from the same amount of vitamin D is significantly intensified when the vitamin is ingested in association with the lactalbumins. Furthermore the vitamin associated with the colloid appears to be quantitatively precipitated, up to certain limits, under the conditions described. The filtrates from which



the precipitated albumin had been removed showed no antirachitic potency at the lower levels and a greatly reduced potency at the higher levels (0.9 microgram of vitamin D per 1.2 mg. of lactalbumin and above). The greatest potency is consistently shown when the vitamin D is associated with the lipid-free lactalbumin.

Data of this character clearly show that the physiological response to unit amounts of vitamin D may vary. Since milk contains an ensemble of proteins in a highly dispersed state, it is reasonable to presume that such a condition would permit a similar accentuation of the effectiveness of the vitamin, if present in the water phase. The conditions under which the experimental lactalbumins were prepared permitted an intimate contact between the dispersed colloid and the vitamin D, both in an aqueous vehicle and in contradistinction to conditions which prevail when the vitamin is carried by an oil vehicle introduced into an aqueous system.

In order to determine whether such increased effectiveness could be demonstrated with milk, the 0.05 per cent solution of vitamin D in propylene glycol was appropriately diluted with water, and the diluted vitamin added to various milks in predetermined amounts. The vitamin was introduced into cold milk, followed by agitation and storage at a low temperature prior to and during the required 10 day feeding period. Like quantities of the vitamin were also fed in water. Since the entire quantity of the different milks required for the experiments was prepared at one time from a single diluted vitamin D solution, mechanical errors which might be introduced by the day to day supplementation with numerous small quantities of milk were entirely avoided. The results shown in Table III<sup>2</sup> are analogous to those obtained

<sup>2</sup> The comparative tests involving the propylene glycol solution of vitamin D are typical of numerous similar experiments in illustrating the enhanced effectiveness of the vitamin when associated with milk and lactalbumin. However, in experiments conducted some months apart, there was a quantitative difference in response from the unit amount of the pure vitamin when fed either alone or with milk or lactalbumin. It was found that this was due to deterioration of the vitamin during storage. When this fact had been clearly ascertained, it was necessary to reestablish the basic response from the pure vitamin before proceeding with confirmatory tests. Fuchs and van Niekerk (13) have recently reported that pure vitamin D in crystalline form deteriorates rapidly.

with lactalbumin. (Figs. 10 to 17 show typical results obtained from unit amounts of vitamin D with and without milk and lactalbumin solutions.) Breast milk which could not be activated to any substantial degree by ultra-violet irradiation enhanced the effectiveness of the vitamin, although not to as great a degree as did cow's milk, either whole or skim.

In order to determine whether the effectiveness of vitamin D is enhanced to any significant degree by milk, when it is carried in an oil vehicle, comparative tests were made with the international

TABLE III

*Antirachitic Response from Unit Amounts of Pure Vitamin D As Influenced by Milk*

Pure vitamin D in aqueous solution incorporated in	Amount of pure vitamin D fed per day	Amount of milk fed per day	Average degree of calcification
	microgram	ml.	+
Water . . . . .	0 06	None	0.0
" . . . . .	0.15	"	0.0
Skim milk . . . . .	0 02	2	0.5
" " . . . . .	0.05	2	1.0
" " . . . . .	0 06	6	1.9
" " . . . . .	0 15	6	3.4
Whole milk . . . . .	0 02	4	0.8
" " . . . . .	0 04	4	1.0
" " . . . . .	0 06	4	2.2
" " . . . . .	0.15	4	2.8
Human milk . . . . .	0.02	4	0.4
" " . . . . .	0.06	4	0.7
" " . . . . .	0 15	4	1.3

standard of vitamin D.<sup>3</sup> Quantities varying from 0.125 to 2 mg. daily were fed and tested in conformity with the United States Pharmacopœia XI specifications (14). The requisite amounts of the standard were diluted with olive oil so that each of the test animals received 0.2 ml. of the dilution daily. Parallel amounts of the standard similarly diluted with olive oil were fed simultaneously with 4 ml. of milk daily, the oil being thoroughly incorporated in

<sup>3</sup> The international standard of vitamin D, Lot 27, was obtained through the courtesy of Dr. E. M. Nelson, Washington, D. C., and Dr. Fullerton Cook, Philadelphia.

the milk. The results (Table IV) show evidence of slightly greater effectiveness of the vitamin D when fed with milk as described; the increase in effectiveness is not as great, however, as when the vitamin D was carried in a water-miscible vehicle.

It is obvious that marked differences in the antirachitic response from unit amounts of vitamin D may be shown by the empirical laboratory methods alone; milk and even an isolated milk constituent, lactalbumin, were shown to enhance the potency of vitamin D under the conditions described. Data of this character would seem to show that "vitamin D units" as they may be

TABLE IV

*Antirachitic Response of Varying Amounts of Vitamin D in Oil (International Standard) As Influenced by Milk*

Amount of international standard fed per day		Average degree of calcification
mg.		+
2	(Without milk)	2 1
2	(4 ml. whole milk)	No data
1 5	(Without milk)	1 6
1.5	(4 ml. whole milk)	1.9
1	(Without milk)	1.4
1	(4 ml. whole milk)	1.9
0.5	(Without milk)	0 7
0.5	(4 ml. whole milk)	1.1
0 25	(Without milk)	0 0
0.25	(4 ml. whole milk)	0.9
0.125	(Without milk)	0 0
0 125	(4 ml. whole milk)	0 2

derived from the line test technique are an inaccurate expression of the amount of vitamin D. It is believed that the numerical unit designation is inapplicable, particularly in the case of irradiated milk, as may be illustrated by the following.

A sample of commercial irradiated milk was fed daily to rachitic rats, prepared in the usual manner, at levels varying from 1.8 to 4 ml., for a period of 10 days. The detailed results recorded in Table V show that the differences in the average calcification values were not in a consistent and proportionate relationship to the varying quantities of milk received by the animals.

The average results from the milk fed at the 4 ml. and the 3.4

ml. levels were identical. If the empirical method were a measure of the true amount of vitamin D received by the animals, there should be some evidence that the 4 ml. quantity of irradiated milk contains more vitamin D than the 3.4 ml. quantity. No such evidence is indicated by the results. A further analysis of the data will also reveal that the evidence does not permit a direct and concrete appraisal of the amount of vitamin D in the different quantities of milk, although it does affirm that the lower quantities have less antirachitic properties than the higher ones.

TABLE V

*Antirachitic Response from Varying Amounts of the Same Irradiated Milk*

Amount of milk fed daily for 10 days	Degree of calcification shown by individual animals										Average degree of calcification
	+	+	+	+	+	+	+	+	+	+	
4	2.0	2.0	2.5	2.0	2.0	1.5	2.0	1.5	1.5		1.9
3.4	2.0	1.5	2.5	2.0	1.5	1.5	2.0	2.0			1.9
2.7	1.5	1.5	1.5	2.0	1.5	1.5	2.0	1.0	1.5		1.6
1.8	1.5	1.0	1.5	1.5	1.5	1.5	1.5	1.0	1.0		1.3

## SUMMARY

The experiments with lactalbumin appear to be significant. When the lactalbumin was precipitated from a solution containing vitamin D in the water phase, the precipitate carried substantially all of the vitamin. Since the precipitate could be thoroughly washed without removing the vitamin, its association with the protein was not merely one of occlusion. Furthermore, since the vitamin carried by the lactalbumin showed a greater potency than that fed alone, it appears that the vitamin D and the lactalbumin had formed a symplex. By definition (15), a symplex is a system consisting of a prosthetic group and a colloidal carrier of high molecular weight. Evidence of such a combination is the greater biological activity of the system than that of the sum of its components. These conditions are fulfilled in the case of the lactalbumin and the vitamin D under consideration. In the light of the above definition, the vitamin D would be considered as the prosthetic group and the lactalbumin as the carrier. It might be added parenthetically that a synthesis of a symplex consisting of

the albumin of blood and cholesterol was recently reported by Rimington (16).

The experiments wherein the lactalbumin containing substantially 7 per cent of lipid matter was compared with lactalbumin devoid of such material seem to emphasize the validity of the above concept. The combination of the lipid-free lactalbumin with the vitamin was found to exhibit a greater antirachitic potency than the lactalbumin-vitamin combination containing lipid matter. In the former, the vitamin D could form a true symplex, the secondary valences (*Restaffinitäten*) of the protein permitting its chemical union with the vitamin. In the latter, the vitamin D merely dissolved in the prosthetic group, namely in the lipid matter associated with the lactalbumin. Such a concept seems to be warranted in view of the data presented, and is further supported by observations of other investigators. Such a concept could explain the high effectiveness of irradiated milk as well as the recent clinical report by Lewis (8), showing that vitamin D administered in the water phase with accompanying milk constituents is more effective clinically than when carried by an oil vehicle.

It is obvious that the biological activity of a substance present in natural products as a prosthetic group of a symplex, or which might form such a system, with appropriate substances, cannot be expressed in units of like value. Considerations of this character must be taken into account in arriving at a sound appraisal of the true value of the empirical laboratory "unit" designation of irradiated milk and other antirachitic agents.

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## EXPLANATION OF PLATES

### PLATE 2

Photomicrographs of typical degrees of calcification taken as reference standards.

- FIG. 1. Negative calcification.
- FIG. 2. 0.5+ degree of calcification.
- FIG. 3. 1.0+ degree of calcification.
- FIG. 4. 1.5+ degree of calcification.
- FIG. 5. 2.0+ degree of calcification.
- FIG. 6. 2.5+ degree of calcification.
- FIG. 7. 3.0+ degree of calcification.
- FIG. 8. 3.5+ degree of calcification.
- FIG. 9. 4.0+ degree of calcification.

### PLATE 3

Photomicrographs of typical specimens showing increased effectiveness of unit amounts of pure vitamin D caused by lactalbumin solutions and milk.

- FIG. 10. 0.02 microgram of vitamin D with 2 ml. of skim milk; 0.5+ degree of calcification.
- FIG. 11. 0.05 microgram of vitamin D with 2 ml. of skim milk; 1.0+ degree of calcification.
- FIG. 12. 0.06 microgram of vitamin D with 6 ml. of water; negative calcification.
- FIG. 13. 0.06 microgram of vitamin D with 1.2 mg. of dispersed lactalbumin; 0.5+ degree of calcification.
- FIG. 14. 0.06 microgram of vitamin D with 6 ml. of skim milk; 2.0+ degree of calcification.
- FIG. 15. 0.15 microgram of vitamin D with 6 ml. of water; negative calcification.
- FIG. 16. 0.15 microgram of vitamin D with 1.2 mg. of dispersed lactalbumin; 1.5+ degree of calcification.
- FIG. 17. 0.15 microgram of vitamin D with 6 ml. of skim milk; 3.5+ degree of calcification.





(Supplee, Ansbacher, Bender, and Flannigan: Milk effect on vitamin D)







(Supplee, Ansbacher, Bender, and Flanigan: Milk effect on vitamin D)



# THE EFFECT OF SOME REAGENTS ON THE "FILTRATE FACTOR" (A WATER-SOLUBLE VITAMIN BELONGING TO THE VITAMIN B COMPLEX AND PREVENTING A DIETARY DERMATITIS IN CHICKS)

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Kline, Keenan, Elvehjem, and Hart (1) have described a syndrome produced in chicks by feeding a heated diet of natural foodstuffs. Elvehjem and Koehn (2) have described the preparation from liver of a concentrate which will prevent the syndrome. The concentrate was prepared from an aqueous extract of liver from which the flavins had been removed by adsorption on fullers' earth and filtration. It is proposed for the sake of brevity to refer in this communication to the factor present in the concentrate as the "filtrate factor," in reference to the method of preparation by its discoverers (1, 2). The name intentionally leaves in abeyance the question of the identity or non-identity of the factor with the rat factor vitamin B<sub>6</sub> (3). It has been suggested by Stare (4) that the two factors may be identical, but György (3) reported vitamin B<sub>6</sub> to be destroyed by visible light, while Elvehjem and Koehn (2) reported that the chick factor was not destroyed by exposure to light.

## EXPERIMENTAL

### *Method of Assay*

The care of the chicks, the production of the syndrome, and the method of scoring for intensity of symptoms have been previously described (5). It was found desirable to introduce the following modifications.

1. Vitamin G (lactoflavin) was added to the diet in the form of a fullers' earth adsorbate of whey corresponding to 60 per cent of

whey. In a few experiments 5 per cent of skim milk powder which had been heated at 120° for 24 hours to destroy the filtrate factor was used instead. This was found necessary because it was shown previously (5, 6) that the basal unheated diet of Elvehjem and coworkers may be deficient in vitamin G (flavin). It is obviously necessary to insure that an experimental diet is as far as possible complete in all essentials except the factor under study.

2. The antihemorrhagic vitamin, which has been demonstrated to be necessary for the chick (7), was added to the diet in the form of a hexane extract of alfalfa meal. The extract was evaporated on the diet. The level fed corresponded to 1 per cent of alfalfa meal. The addition abruptly checked a tendency to hemorrhagic symptoms which had been occasionally observed. The hemorrhagic symptoms may have been aggravated by the fact that the birds were rigorously excluded from access to their droppings, since Almquist and Stokstad have observed (unpublished data) that the antihemorrhagic vitamin is present in chick droppings.

3. The period on the stock diet (5) was 10 days, and the depletion period on the diet heated at 120° was from 5 to 12 days. Chicks appeared to be less vigorous in the summer and fall months, and a shorter depletion period was used during these times.

The birds were weighed individually and symptoms noted at intervals during the assay period of 2 weeks. The average gain per bird for a period of 2 weeks on the supplemented diet was compared with the gain by the control group on the basal diet. Ten birds were used in each group. The "syndrome score" (5) was roughly correlated with weight changes but was found to be too variable for use in assaying the filtrate factor.

#### *Experiments with Crude Liver Filtrate*

Extract of beef liver (5) was shaken with fullers' earth to remove vitamins B and G, and filtered. The liver filtrate was used for the following experiments.

*Effect of Autoclaving Followed by Treatment with Fullers' Earth*—A sample of liver filtrate was autoclaved at its natural pH of about 5 at 20 pounds pressure for 30 minutes, and shaken with fullers' earth and filtered. The fullers' earth was inactive, and the potency of the filtrate was undiminished.

*Extraction with Butyl Alcohol*—A sample of liver filtrate was

acidified with hydrochloric acid and submitted to continuous extraction with butyl alcohol in the apparatus described by Dakin (8). The factor was extracted, but the process was slow, and was accompanied by darkening.

*Attempt with Adsorption with Lead Sulfide*—A sample of liver filtrate was treated with lead chloride and hydrogen sulfide. The precipitate of lead sulfide was removed by filtration. The potency of the filtrate was undiminished, and the filtrate factor could not be detected in a sodium hydroxide eluate of the lead sulfide precipitate.

*Experiments with Concentrated Extract Prepared from Liver Filtrate*

It has been shown by Elvehjem and Koehn (2) that the filtrate factor distributes itself between amyl alcohol and water in a proportion governed by the pH of the aqueous phase. This observation was made use of in devising a method for preparing concentrated solutions of the factor. Liver filtrate was brought to a pH of between 1 and 2 and shaken with repeated changes of isoamyl alcohol. The combined amyl alcohol layers were shaken with three changes of dilute sodium hydroxide to extract the factor. The resultant aqueous solution was brought to a pH of about 4 and concentrated under reduced pressure to a convenient volume. The solution was added to the heated diet and assayed biologically. Aliquots of the solution were then submitted to chemical treatments, and fed at a level which was usually twice as great as the level of the original solution found necessary to produce the maximum growth response.

*Precipitation of Impurities with Barium Hydroxide*—Excess of saturated barium hydroxide solution was added and the mixture allowed to stand overnight at 0°. The precipitate was separated, and the precipitate and the filtrate were fed separately after removal of barium with sulfuric acid. The precipitate did not contain the factor, which passed into the filtrate without perceptible loss. The same result was obtained when barium hydroxide precipitation was made in 80 per cent alcoholic solution, which led to a removal of an even greater amount of inert material.

*Fractionation with Lead Acetate*—60 cc. of concentrate were diluted to 200 cc. and 60 cc. of saturated lead acetate solution were added. The mixture was cooled with ice and ammonium hydrox-

ide was slowly added, with stirring, until a pH of 6.6 was reached. The precipitate was removed, dissolved in acetic acid, and reprecipitated at pH 6.6. The combined filtrates were brought to pH 8.0, and the precipitate removed, dissolved in acetic acid, and reprecipitated at pH 8.0. The filtrates were combined, and all fractions were freed from lead by addition of a little hydrochloric acid and excess of hydrogen sulfide. The factor was absent from the precipitates at pH 6.6 and pH 8.0, and present in the filtrate from precipitation at pH 8.0, although about half of the potency had disappeared.

*Attempts at Inactivation with Oxidizing Agents. Nitrous Acid*—30 cc. of concentrate were acidified with HCl and placed in a boiling water bath. 15 cc. of 5 per cent sodium nitrite were added over a period of 20 minutes, followed by 15 cc. of 7.5 per cent urea solution. A slight diminution in potency was brought about by the treatment. Another aliquot of the concentrate was diluted with 1 volume of concentrated HCl, and cooled to 0°. 1 volume of 35 per cent sodium nitrite solution was added, the temperature being kept below 5°. The mixture was allowed to stand overnight at 0°, and excess of urea was then added. The solution was aerated at room temperature and boiled at 40° under reduced pressure. A slight diminution in potency was brought about by the treatment.

*Bromine*—2.5 volumes of saturated bromine water were added to the concentrate. The mixture was allowed to stand for 1 hour at room temperature and then aerated to remove free bromine. The precipitate was discarded, and the filtrate was fed. No diminution in potency was detected. The experiment was repeated, but the bromine was removed by aeration at 60°. A slight diminution in potency was found.

*Nitric Acid*—4 cc. of nitric acid were added to 30 cc. of the concentrate. The mixture was heated to 60° and allowed to cool spontaneously. It was neutralized with sodium hydroxide and fed. No diminution in potency was detected.

*Attempt at Adsorption with Ferric Hydroxide*—Three aliquots of 20 cc. of the concentrate, Samples 1, 2, and 3, were used. To Samples 1 and 2, 0.75 gm. of ferric chloride were added, and to Sample 2 was added a slight excess (14 cc.) of 1 N sodium hydroxide solution. An equal amount of sodium hydroxide solution was

added to Sample 3. All three aliquots were heated in a boiling water bath for a few minutes to flocculate the precipitate in Sample 2. The precipitate and filtrate in Sample 2 were separated, the precipitate redissolved in dilute hydrochloric acid, and the filtrate acidified. Sample 3 was acidified, and all solutions were fed. Ferric chloride alone caused no inactivation. Sodium hydroxide alone caused a partial inactivation. No potency was detected in the filtrate from ferric hydroxide precipitation, or in the redissolved ferric hydroxide precipitate.

*Attempt at Adsorption with Charcoal*—An aliquot of the concentrate was treated with acid-washed norit according to Kinnersley *et al.* (9), with 12 gm. of charcoal per 100 cc. The filtrate from charcoal was colorless and potent. The yellowish acid-alcohol eluate ("Peters' eluate") of charcoal was not potent.

*Behavior of Charcoal Filtrate towards Oxidizing and Reducing Agents*—Separate aliquots of the charcoal filtrate of the preceding paragraph were treated with cold bromine water, with 0.2 volume of 30 per cent hydrogen peroxide solution, and with sodium bisulfite according to Williams *et al.* (10). In no case could reduction of potency be detected.

#### *Removal of Inert Material from Aqueous Extract of Rice Bran*

Concentrated aqueous rice bran extract (5), freed from vitamins B and G by treatment with fullers' earth, was diluted with 10 volumes of methanol and the resultant precipitate discarded. The filtrate was concentrated *in vacuo* to small bulk. A portion of this aqueous solution was mixed with 2 volumes of methanol and 6 volumes of acetone to precipitate inert material. The factor remained in solution. Another portion was mixed with 2 volumes of methanol and 3 volumes of secondary amyl alcohol. The factor remained in solution.

#### *Demonstration of Deficiency of Filtrate Factor in Unheated Diet of Purified Foodstuffs*

8 day-old chicks were fed a diet of starch, washed casein, vitamin B adsorbate prepared by shaking rice bran extract with fullers' earth, vitamin G adsorbate prepared similarly from whey, paper pulp, salt mixture (4), and cod liver oil. Growth was slow, and typical dermatitis appeared in about 10 days. Liver filtrate was



then added to the diet, and growth improved almost immediately. Dermatitis began to disappear, but within 3 weeks nearly all of the chicks were dead, owing to lack of the antiencephalomalacic vitamin (11, 12).

#### DISCUSSION

The filtrate factor differs markedly from vitamins B and G in its failure to be adsorbed readily from acid solution by fullers' earth or charcoal. In contrast to vitamin B, it is not destroyed by autoclaving; while in contrast to vitamin G, it is destroyed by dry heat at 120° for 24 hours, which does not destroy vitamin B (13). The factor is noteworthy for its resistance to oxidizing and reducing agents and its solubility in moist, weakly polar solvents.

Dermatitis was produced on an unheated diet of purified foodstuffs and cured by the filtrate factor. The diet was incomplete, but the experiment served to emphasize that the dermatitis caused by the heated diet is due to a deficiency rather than to production of injurious substances in the diet of natural foodstuffs by the heat treatment.

Block and Hubbell (14) have recently presented evidence that the third factor of the vitamin B complex, essential for the rat, is adsorbed on Lloyd's reagent and is eluted by dilute sodium hydroxide. Experiments completed in this laboratory confirm this result, and indicate that an extract of rice bran may be separated into two fractions by means of treatment with fullers' earth; the unadsorbed fraction is more potent for the chick, when fed with the heated diet, and the adsorbed fraction is more potent for the rat.

We prefer to retain the nomenclature vitamin G for flavin, in common with Bisbey and Sherman (15) and with Block and Hubbell (14). Experiments with crystalline lactoflavin and hepato-flavin (6) have indicated that the growth-promoting effect of whey adsorbate and liver adsorbate when added to chick diets adequately supplemented with the filtrate factor is due to vitamin G (flavin).

#### SUMMARY

1. The name "filtrate factor" is provisionally applied to the water-soluble vitamin which has been demonstrated by workers at

the University of Wisconsin to prevent the dermatitis produced in chicks by feeding a heated diet of natural foodstuffs (1).

2. The factor was not precipitated by barium hydroxide in aqueous or 80 per cent alcoholic solution, and was not destroyed by bromine water, ferric chloride, dilute nitric acid, sodium bisulfite, hydrogen peroxide, or hydrogen sulfide. Nitrous acid brought about a slight diminution in potency. The factor was partially inactivated by warming with sodium hydroxide, and completely inactivated by warming with a mixture of ferric chloride and sodium hydroxide.

3. Norit, lead sulfide, and fullers' earth failed to adsorb the factor appreciably from acid solution.

4. Fractional precipitation with lead acetate failed to yield the factor in the precipitates at pH 6.6 and pH 8.0, and a large part of the original potency was found in the filtrate from the precipitation at pH 8.0.

5. Concentrated solutions of the factor were prepared from an aqueous extract of rice bran by treatment with fullers' earth, methanol, and acetone or secondary amyl alcohol.

6. Dermatitis in chicks was also produced by feeding a purified diet, and cured by addition of the filtrate factor to the diet.

7. Indications have been found that, by means of treatment of an aqueous extract of rice bran with fullers' earth, the third factor of the vitamin B complex, essential for the rat, may be at least partially separated from the filtrate factor, which supplements the heated diet for the chick.

Our thanks are due to Vitab Products, Inc., of San Francisco, for supplying us with whey adsorbate (used as a source of vitamin G) and rice bran extract; to Armour and Company, Chicago, for liver extract; to the California Milk Products Company, Gustine, for casein; and to the F. E. Booth Company for the loan of battery brooders. The assistance of K. K. Miya and S. Grushkowitz is gratefully acknowledged. Facilities of the Animal Husbandry Division were kindly placed at our disposal by Dr. George H. Hart.

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# THE DISTRIBUTION OF THE "FILTRATE FACTOR" (A WATER-SOLUBLE VITAMIN BELONGING TO THE VITAMIN B COMPLEX AND PREVENTING A DIETARY DERMATITIS IN CHICKS) IN CERTAIN FEEDINGSTUFFS

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The present communication gives an account of the distribution in certain natural feedingstuffs of a water-soluble vitamin ("filtrate factor"), preventing or curing a dermatitis in chicks (1, 2).

The method of assay of the filtrate factor was reported in the preceding article (3).

The feedingstuffs tested were incorporated into the basal heated diet in a manner calculated to disturb its nutritive ratio as little as possible; *e.g.*, 80 per cent of wheat was substituted for 55 per cent of heated corn and 25 per cent of heated wheat middlings, while in another case 25 per cent of skim milk powder was substituted for 8 per cent of heated casein and 17 per cent of heated corn.

Many of the feedingstuffs tested could not be fed at a level high enough to provide sufficient filtrate factor for maximal growth. It was hence necessary to determine the effect of suboptimal amounts of the factor upon growth. This was done by means of addition to the heated diet of various levels of concentrates prepared from liver. The results of such an assay are shown in Fig. 1.

The results of the experiment illustrated in Fig. 1 and of several other such experiments indicated that growth response to suboptimal amounts of the filtrate factor was roughly proportional to the amount present. This finding was applied to the assay of the factor in natural feeds. Most feeds could not be fed at a level sufficiently high to provide enough of the factor for maximal growth. In each series a group was included on the basal diet, and a group on a diet consisting of the basal diet *plus* an excess of

TABLE I  
*Filtrate Factor Values of Some Natural Feeds*

Material	Per cent fed in basal ration	Filtrate factor value	Mean filtrate factor value
Brewers' Yeast 1.....	3	16	
"    "    1.....	8	10	13
Autoclaved Brewers' Yeast 1.....	2.5	12	
"    "    "    1.....	8	12	12
Brewers' Yeast 2*.....	6	11	11
Bakers'    "    1*.....	1.7	13	
"    "    1.....	6	17	
"    "    1.....	15	>7†	15
Autoclaved Bakers' Yeast 2.....	4	1.3	
"    "    "    2.....	8	2.0	1.6
Hawaiian cane molasses.....	5	7	
"    "    ".....	8	7	
"    "    ".....	15	>6 7†	7
Spray-dried whey powder.....	15	4	
"    "    ".....	30	>3.3†	4
Roller-dried skim milk powder.....	5	2 7	
"    "    "    "    ".....	12	2 2	
"    "    "    "    ".....	17	3 1	
"    "    "    "    ".....	18	2 8	
"    "    "    "    ".....	25	3.1	3
Dehydrated Alfalfa Meal 1.....	8	4	
"    "    "    1.....	10	2	3
"    "    "    2.....	10	1 0	
"    "    "    2.....	15	2 2	1.6
Dried kale.....	8	2.8	
"    ".....	15	1.8	2
Fresh    "    fed <i>ad libitum</i> .....	50 (Ca.)	0 6	
"    "    "    "    ".....	50 "	0 9	0.8
Ground whole wheat.....	80	0 8	
"    "    ".....	80	0 8	0.8
Wheat middlings.....	25	0.7	
"    ".....	80	0.8	0.8
"    germ.....	25	0 2	
"    ".....	40	0.9	
"    ".....	60	0 3	0.5
"    Bran 1.....	30	1.7	
"    "    1.....	50	2.0	1.8
Ground whole barley.....	80	0.7	
"    "    ".....	80	0.7	0.7
"    "    corn.....	58	0.5	
"    "    ".....	80	0.7	
"    "    ".....	80	0 5	0.6

\* Supplied by courtesy of Standard Brands, Inc.

† The level fed was supraoptimal.

TABLE I—*Concluded*

Material	Per cent fed in basal ration	Filtrate factor value	Mean filtrate factor value
Polished rice.....	80	0.33	
“ “.....	80	0.27	0.3
Rice bran.....	30	1.1	
“ “.....	50	1.9	1.5
Meat Scrap 1.....	24	0.4	
“ “ 2.....	24	<0.2	0.3
Fish-meal.....	18	<0.2	
“ “.....	20	<0.2	<0.2
Commercial casein†.....	12	<0.2	
“ “.....	12	<0.2	<0.2
Dilute sulfuric acid extract of Wheat Bran 1.....	Equivalent to 20	2	2

† Furnished generously by the California Milk Products Company, Gustine.

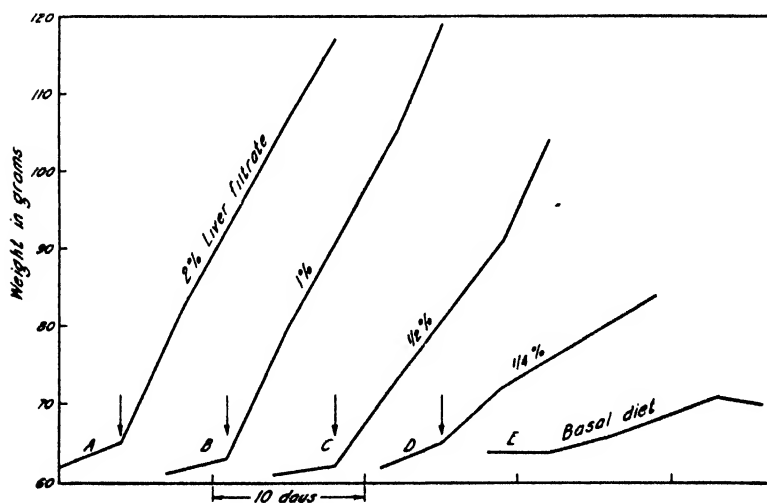


FIG. 1. Roughly arithmetical proportion between growth response and level of filtrate factor was obtained until a level of 1 per cent liver filtrate was reached. A further increase of filtrate produced no further increase in growth. The syndrome score (4) at end of the supplementation period was for the group represented by Curve A, 1; Curve B, 4; Curve C, 19; Curve D, 32; Curve E, 37.

the filtrate factor supplied as a concentrate prepared from liver or from rice bran. The group on this positive control diet gave a measure of the maximal net growth response, and other groups on test rations usually gave growth responses intermediate between that of the positive control group and the group on the basal diet. The findings illustrated in Fig. 1 made it possible to apply the following formula to calculate the filtrate factor value of the feeds studied.

$$\text{Filtrate factor value} = \frac{100 \times (\text{growth on test diet} - \text{growth on basal diet})}{(\text{Growth on positive control diet} - \text{growth on basal diet}) \times \text{per cent of feed added to test diet}}$$

*Example—*

	Gain in 14 days gm.
Basal diet . . . . .	1
Positive control diet . . . . .	41
Basal diet with 40 parts unheated wheat germ replacing 25 parts heated wheat middlings and 15 parts heated corn-meal . . .	15

$$\text{Filtrate factor value of wheat germ} = \frac{100 \times (15 - 1)}{(41 - 1) \times 40} = 0.9$$

Table I contains a list of the filtrate factor values of the feeds studied. Each value represents the average of at least two experiments. Each series was treated separately in making the calculations, since the response varied with the season of the year.

#### DISCUSSION

It is possible to arrive at a rough estimate of the adequacy of a chick ration with respect to the filtrate factor by multiplying the percentage of each constituent by its filtrate factor value and adding the products. If the sum is less than 1.0, a deficiency of the filtrate factor in the diet is indicated.

The requirements of the chick for the filtrate factor appear to vary with age. It was observed several times that chicks on certain simplified diets developed dermatitis at 6 weeks of age and that the dermatitis disappeared at 8 weeks of age. The requirements, expressed as a percentage of the diet, appeared from this observation to be greater at 7 weeks of age than at 4 weeks of age, since the filtrate factor had been included in the diet at a level

found to produce maximal growth by the usual method of assay on chicks between the ages of 3 weeks and 5 weeks.

The observation that wheat germ and fresh kale are about the same as corn as a source of the filtrate factor is in sharp contrast to the great superiority of wheat germ and canned kale to corn as a source of the human P-P factor (5), and suggests that the two factors may not be identical.

#### SUMMARY

1. Growth response to suboptimal levels of the "filtrate factor," a dietary essential for the chick, belonging to the vitamin B complex, was roughly proportional to the amount of the factor which was fed.

2. A method is described for roughly calculating the level of the factor in the diet from the growth response.

3. The relative values of several common feedingstuffs with respect to the factor are tabulated.

4. The probable non-identity of the filtrate factor with the P-P factor is briefly discussed,

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## NOTE ON THE PREPARATION OF SINIGRIN\*

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Sinigrin,  $C_9H_8N \cdot C(S \cdot C_6H_{11}O_5) \cdot OSO_3K$ , the important sulfur-bearing glycoside of black mustard, *Brassica nigra*, has been known for almost a century (1). From the procedure given in the standard phytochemical compilations, one would conclude that its preparation is a relatively simple matter (2-4). However, we were surprised to discover that the procedure usually advised, which dates back to the classical researches of Gadamar on the constitution of sinigrin (5), yielded negative results. On the other hand, excellent results were obtained by the method of Herissey and Boivin (6), which is usually cited in the handbooks as an *alternative* to Gadamar's preparation.

Dr. W. Schneider, of the University of Jena, has informed us that about 20 years ago Gadamar's method was employed successfully in his laboratory (by F. Wrede), but that in recent years it could not be repeated.<sup>1</sup> He also informed us that E. Merck

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<sup>1</sup> In connection with the studies on myrosin, the specific enzyme that hydrolyzes the naturally occurring mustard oil glycosides, both Neuberg and Wagner (7) and von Euler and Eriksson (8), as late as 1926, reported that the sinigrin used was prepared by Gadamar's (5) method. On the other hand, Heiduschka and Pyriki (9) as well as Herissey and Boivin (6) found in confirmation with our observations that this method yielded negative results. Recently, the successful preparation of sinigrin with Herissey and Boivin's procedure was reported by Sandberg and Holly (10); these investigators made no mention of Gadamar's method.

of Darmstadt had had a similar experience. We might add that attempts to purchase sinigrin, both in this country and abroad, failed, although prior to 1914 it was usually obtainable from pharmaceutical houses.

Herissey and Boivin (6) pointed out that the difficulties encountered in Gadamar's method were caused by contamination with large quantities of sucrose. In corroboration, the only crystalline product that we could obtain with Gadamar's method was sucrose.<sup>2</sup> The essential point in the improved method (6, 10) is that the sugars are removed by fermentation with yeast.

Since the standard phytochemical handbooks (2-4) emphasize a method that is obsolete and unreliable, we trust that the above will be of value to other investigators requiring sinigrin.

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<sup>2</sup> An attempt to prepare sinalbin from white mustard seeds by Gadamar's method (5) also yielded only sucrose.

## THE RELATIONSHIP BETWEEN TOTAL AND FREE CHOLESTEROL IN HUMAN BLOOD SERUM\*

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During the past 2 years the method of Schoenheimer and Sperry (1) has been employed in this laboratory for determining the concentration of total and free cholesterol in approximately 1500 samples of blood serum from healthy and diseased human subjects. The outstanding result has been the finding that in healthy adults, and probably also in children (except in the neonatal period), the proportion of the cholesterol in the free form is remarkably constant, despite large differences in the total amount present. With few exceptions significant variations have been observed only in diseased individuals (especially with infections and diseases of the liver) and in infants during the neonatal period (2), and the conclusion has been reached that the relationship between the concentrations of total and free (or combined) cholesterol is a far more valuable index of changes from the normal cholesterol metabolism than the absolute amounts in the serum.

### EXPERIMENTAL

The concentration of total and free cholesterol was determined in 126 samples of blood serum from 91 healthy, adult human subjects (Table I). Half of the samples were taken from 59 persons before breakfast specifically to establish the range of normal values given by the method of Schoenheimer and Sperry. The remaining determinations were made in connection with other investigations and no attention was paid to the proximity of the

\* This investigation was made possible by the support of the Josiah Macy, Jr., Foundation.

TABLE I

*Cholesterol in Blood Serum of Healthy Adult Human Subjects*

Subject No.	Age	Total cholesterol	Free in total cholesterol	Subject No.	Age	Total cholesterol	Free in total cholesterol
Before breakfast							
	yrs.	mg. per 100 cc.	per cent		yrs.	mg. per 100 cc.	per cent
1-a	33	195.2	26.9	29	22	204.6	26.4
1-b	33	195.7	26.0	30	22	228.0	24.5
1-c	33	208.5	24.5	31	21	154.2	24.3
2-a	24	334.3	26.9	32	19	168.5	26.3
3-a	35	240.5	28.1	33	23	168.4	25.6
4	35*	165.8	28.1	34	22	205.0	25.5
5	23	195.0	27.6	35	24	188.2	26.6
6-a	35	131.5	26.9	36	24	184.0	27.4
7-a	36	269.0	27.8	37	29	205.1	25.5
8-a	26	207.0	26.1	38	23	176.0	24.8
8-b	26	213.1	26.0	39	22	230.0	26.6
9-a	42	249.5	26.0	40†	35	222.4	27.2
9-b	42	250.0	26.2	41	21	167.3	25.3
10	25	155.5	25.4	42	22	206.5	24.6
11	24	224.0	24.4	43	23	148.4	25.4
12	26	158.2	25.5	44	25	221.2	26.7
13†	19	217.0	25.9	45	24	227.3	26.3
14	22	139.3	28.4	46	22	264.0	28.7
15†	21	168.4	29.3	47	20	189.6	26.5
16	24	255.2	25.6	48	19	217.1	26.5
17	22	157.4	26.9	49	23	190.2	27.7
18	20	213.5	26.2	50-a	23	325.5	25.1
19	23	193.5	27.2	51†	21	172.7	25.6
20	24	216.9	27.8	52†	22	219.6	24.9
21	24	240.6	25.4	53	27	169.5	25.7
22	22	135.8	27.9	54	22	182.5	25.2
23	21	225.4	26.1	55	22	184.0	27.6
24	26	205.0	28.3	56	23	153.2	24.6
25	23	140.6	24.4	57†	21	160.0	26.8
26	20	215.2	25.3	58	26	216.5	27.5
27	21	156.8	24.9	59	21	156.4	25.6
28	20	181.4	25.7	Average . . . . .			26.3

\* Approximate age.

† Female subjects.

TABLE I—*Concluded*

Subject No.	Age	Total cholesterol	Free in total cholesterol	Subject No.	Age	Total cholesterol	
	<i>yrs.</i>	<i>mg. per 100 cc.</i>	<i>per cent</i>		<i>yrs.</i>	<i>mg. per 100 cc.</i>	<i>per cent</i>
1-d	34	187.0	28.9	63	23*	248.5	26.8
1-e	34	209.0	26.1	64	33	243.6	27.3
1-f	34	188.3	27.6	65	27*	392.0	26.2
1-g	34	201.5	27.5	66	25*	188.0	26.0
1-h	34	216.7	28.0	67	23	164.0	26.3
1-i	35	196.5	26.7	68†	26	152.9	28.1
2-b	24	322.5	27.5	69-a†	30	177.5	26.5
2-c	24	329.0	29.6	69-b†	30	146.0	25.7
2-d	25	253.0	29.8	70-a†	26	201.3	27.6
2-e	25	321.5	29.3	70-b†	26	189.8	27.2
3-b	35	216.3	29.1	71-a†	21	168.5	25.5
3-c	35	220.3	27.4	71-b†	21	168.5	28.1
3-d	35	223.3	27.0	72†	33	179.5	27.8
3-e	36	199.3	27.3	73†	30*	171.0	28.0
3-f	36	253.5	28.2	74†	31*	229.3	27.3
3-g	36	239.3	26.0	75	34	282.8	27.5
6-b	36	140.5	25.3	76	25*	151.3	26.0
7-b	36	264.0	28.4	77	32	202.3	26.2
7-c	36	247.0	30.1	78	23*	239.3	29.1
7-d	37	232.8	29.4	79	21*	151.5	29.3
7-e	37	229.8	28.1	80	22*	156.8	28.3
8-c	27	200.3	26.6	81	23*	220.3	26.2
9-c	43	266.8	26.3	82	39	249.5	28.5
50-b	24	305.3	25.4	83†	38	197.8	26.0
60-a	19	186.8	28.6	84	26	225.3	26.9
60-b	19	160.5	28.6	85	23	175.8	25.9
61-a†	26	336.0	25.4	86†	40	191.0	26.9
61-b†	26	319.0	27.4	87	30	175.8	29.1
61-c†	26	359.0	27.0	88†	30	195.8	29.4
62-a	29	233.7	26.1	89	33	185.9	28.3
62-b	29	192.0	29.5	90	23*	198.5	27.2
				91	25	204.5	27.2
				Average . . . . .			27.5
Average of all determinations . . . . .					209.8	26.9 ± 1.4 (S.D.)	

last meal. They are included with the fasting results as normal values, because most of the reports in the literature and some experiments carried out in this laboratory indicate that in humans neither the total cholesterol content of the blood serum or plasma nor the proportion between the various cholesterol fractions is altered significantly after ordinary meals.<sup>1</sup> Boyd's recent results (6) are particularly conclusive in showing no appreciable change in the average concentration of any of the cholesterol fractions during the day or night in persons following their usual routine.

The author determined the total and free cholesterol content of the serum in several subjects before and at frequent intervals after a breakfast which in some cases was abnormally rich in cholesterol (three eggs, six slices of bacon, two slices of toast, heavily buttered, two cups of coffee with cream, and orange juice). Only small variations in the total cholesterol concentration in both directions (maximum 12 per cent) were observed, the average change, 3 to 4 hours after eating, in ten such experiments being zero. Equally large variations were found in some of the same subjects while fasting. There was no significant change in the proportion of the total cholesterol in the free form in any case.

The data set forth in Table I show that the percentage of free in total cholesterol, as determined by the method of Schoenheimer and Sperry, is remarkably constant in the blood serum of healthy, adult humans. The lowest value found was 24.3 and the highest was 30.1 per cent.

Support for the foregoing conclusion was obtained in the course of an investigation<sup>2</sup> during which the concentration of total and free cholesterol was determined in 150 samples of blood serum taken post mortem from individuals who had died suddenly from various causes, usually trauma. In 115 instances the per-

<sup>1</sup> Bürger and Habs (3) stated that large increases, up to 100 per cent, in the amount of cholesterol in the serum followed the ingestion of the highly unphysiological meal of 100 gm. of olive oil and 5 gm. of cholesterol, but Snapper and Parisel (4) and Barreda (5) were unable to confirm this finding. Barreda found a small decrease more often than a small increase in a considerable series of well controlled experiments with the test meal of Bürger and Habs.

<sup>2</sup> A detailed report of this investigation, which was carried out for another purpose, will be published later by Dr. Kurt Lande, Dr. Rudolf Schoenheimer, and the author.

centage of free in total cholesterol was within the normal range (24 to 30 per cent), in fourteen instances below 24 per cent, and in twenty-one instances above 30 per cent. The finding of some values slightly below the normal range (twelve out of fourteen were between 22 and 24 per cent) may be attributed with high probability to esterification *in vitro*, which is to be expected (7) when there is unavoidable delay in obtaining the blood and transporting it to the laboratory. In ten of the twenty-one subjects with percentages over 30 per cent, infection (usually pneumonia) was found at autopsy, while three subjects had fatty livers. In the remaining eight cases there was no evidence of any disease which might account for the high percentage of free cholesterol. In most of these the variation from the normal range was not large (seven out of eight were below 37 per cent), and it is not surprising, in view of the extreme conditions of these experiments, that some abnormal values were found. The fact that such a large proportion of the samples gave the same result as in healthy living subjects emphasizes the constancy of the percentage of free in total cholesterol in adult human blood serum.

In children as in adults the percentage of free in total cholesterol appears to be relatively constant, although as yet too few determinations have been made to establish with certainty the normal range of variation in health in different age groups (except in the neonatal period, in which the percentage has been found to be higher on the average and to be much more variable than in adults (2)). In 45 normal children, 10 months to 13 years old, the percentage of free in total cholesterol varied from 25.7 to 31.0 per cent.

A much larger series of determinations was made on infants (beyond the neonatal period) and children, most of whom were patients of the Babies Hospital or Vanderbilt Clinic, and most of whom were diseased. Approximately four-fifths of 958 such analyses (on 302 patients) gave values between 24 and 31 per cent free in total cholesterol. The finding that such a large proportion of the results from diseased children fell in approximately the same small range observed in healthy adults lends strong support to the view that the percentage of free in total cholesterol is a physiological constant of considerable importance.

In 179 samples of serum (18.7 per cent of the total number



in the above series) from 112 children the percentage of free in total cholesterol was above 31 per cent (tentatively taken as the upper limit of normal in children). There was a history of liver disease, infection, or both at the time all but eight of these samples were taken.<sup>3</sup> In the eight exceptions (from seven subjects) the values were only slightly above 31 per cent, the maximum being 34.5 per cent and the average 32.7 per cent, as compared with an average of 43.5 per cent for the remaining 171 samples. The finding of an increased proportion of free cholesterol in liver disease is in agreement with several previous reports. Epstein (8), in particular, has made an extensive study of the phenomenon. That the percentage of free in total cholesterol tends to rise in the presence of infection was noted by Dr. John D. Lyttle and the author, who followed the cholesterol of the serum in several children with scarlet fever.<sup>4</sup> Stoesser (9) recently reported an increase in the proportion of free in total cholesterol of the blood plasma during acute infections of the respiratory tract and pneumonia.

#### DISCUSSION

In 1917 Bloor and Knudson (10) called attention to the relative constancy of the proportion between free and combined cholesterol in normal human blood plasma. The data on which their suggestion was based were, however, far more variable than those obtained in the present investigation, ranging from 30.0 to 54.3 per cent of free in total cholesterol with an average of about 40 per cent. Most authors have reported normal values similar to those found by Bloor and Knudson. A detailed discussion of these results would require too much space, but a recent publication of Page, Kirk, Lewis, Thompson, and Van Slyke (11) deserves special mention because it presents the largest series of such determinations in the literature. The method of Kirk, Page, and Van Slyke (12) was employed in determining the concentration of total and free cholesterol, along with other lipid constituents, in heparinized blood plasma from 67 healthy men varying from 21 to 101 years old. The percentage of free in total cholesterol,

<sup>3</sup> The author is indebted to Dr. John D. Lyttle for his interpretation of the case histories.

<sup>4</sup> A preliminary report of this investigation was presented in May, 1934, before the Society for Pediatric Research at Atlantic City.

calculated from their data, varied from 22.4 to 72.3 per cent, and the average (Subjects 24, 54, and 67 being omitted) was 37.0 per cent, with a standard deviation of 9.9 per cent. These results stand in marked contrast to those presented in Table I, both in regard to variation of the individual data and higher average percentage of free in total cholesterol.

The discrepancy cannot be due to the use of heparinized plasma by Page *et al.* and serum here, because it has been shown that both preparations give the same cholesterol values (13). It is also not to be explained by the difference in average age of the two groups of subjects, since no significant relation between age and cholesterol concentration was found (11). If the comparison be made on the basis of the age group (20 to 39 years) comparable to that studied here, the inconsistency becomes even greater, since, in this group, the percentage of free in total cholesterol varied from 32.3 to 72.3 per cent with an average of 42.4 per cent. Furthermore, the lack of agreement can hardly be attributed to differences in completeness of extraction or in the determination of total cholesterol, as approximately the same range of total cholesterol concentrations was found by Page *et al.* (109 to 376 mg. per 100 cc.) as by the author (132 to 392 mg. per 100 cc.). Moreover, if a loss of total cholesterol occurred in the procedure of Schoenheimer and Sperry, the apparent percentage of free cholesterol would be too high.

The discrepancy appears to be associated with errors in the determination of free cholesterol. Page *et al.* noted that the average concentration of free cholesterol which they found was considerably higher than had been reported by some other authors and attempted, without success, to discover an error in their procedure which would account for the difference. The author has been equally unable to discover an error in the procedure of Schoenheimer and Sperry of sufficient magnitude<sup>5</sup> to account

<sup>5</sup> The probable error of measurement, calculated from duplicate analyses on forty-six samples of serum, was 1.56 mg. per 100 cc. for total cholesterol, and 0.77 mg. per 100 cc. for free cholesterol. From these results it may be computed that within the normal range the probable error of measurement of the percentage of free in total cholesterol is 0.44 per cent. The high reproducibility indicated by this result does not exclude the possibility of a systematic error in the method of Schoenheimer and Sperry but it does show that random errors of large magnitude do not occur.

for the differences observed. If the proportion of free cholesterol is actually as high on the average and varies as much as the results of previous workers indicate, it follows that large and variable losses of free cholesterol must occur in the procedure of Schoenheimer and Sperry in such a manner as to indicate a relatively constant proportion of total and free cholesterol. The probability of such a chance occurrence is extremely remote.

#### SUMMARY

The concentration of total and free cholesterol was determined in 126 samples of blood serum from 91 healthy, adult, human subjects. The minimum amount of free in total cholesterol was 24.3 per cent, the maximum was 30.1 per cent, and the average was 26.9 per cent, with a standard deviation of 1.4 per cent. The result leads to the conclusion that the percentage is far more constant than has been recognized by previous investigators, most of whom have reported much larger variations and a higher average. Support for this conclusion was obtained in the finding that a large proportion of samples taken post mortem from humans who had died suddenly gave values in the same narrow range. Similar results were obtained in healthy children and in four-fifths of a large series of determinations on diseased children. With few exceptions values above 31 per cent were found only in the presence of infection or liver disease.

The percentage of free in total cholesterol appears to be a physiological constant which may be of considerable value in the study of cholesterol metabolism and in pathology.

Grateful acknowledgment is made to Mr. V. A. Stoyanoff for his invaluable technical assistance in this investigation.

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# VARIATIONS IN THE MAGNESIUM CONTENT OF THE NORMAL WHITE RAT WITH GROWTH AND DEVELOPMENT

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In connection with the investigation on the biochemical effects of magnesium deprivation now being prosecuted by the authors, data were obtained on the water and magnesium content of normal rats at different stages of their growth and development. Since these magnesium values are somewhat at variance with the data which have been published by Buckner and Peter (1), and by Medes and Humphrey (2), it is of interest briefly to present our results.

According to the work of Buckner and Peter, while there is a considerable individual fluctuation, the percentage magnesium content remains virtually unaltered during the whole life period of the rat. Their figures also appear to indicate that the females have a slightly higher magnesium content than the males.

From the data of Medes and Humphrey it may be deduced that the magnesium level is highest at birth, falls to a minimum at 30 days of age, and then increases to reach a maximum again at the age of 70 to 80 days. The existence of certain sex differences in magnesium content is also suggested by Medes and Humphrey.

The variance from these results obtained in the present work will be presented after the experimental procedures are described.

## EXPERIMENTAL

The water content of the animals was obtained by drying the carcasses in an electric oven to constant weight at the temperature of 90°. The carcasses were prepared for magnesium analysis and the analysis carried out in the manner described by Greenberg,

Anderson, and Tufts (3). Of the material analyzed, the animals up to 4 weeks of age were suckling young, taken from mothers maintained on the stock colony diet. Those older than this were mainly control animals from the work on magnesium deficiency. These rats were reared on a synthetic diet consisting of casein, sucrose, hydrogenated cottonseed oil, a salt mixture, and vitamin supplements in the form of rice bran extract, liver extract, and cod liver oil. The magnesium content of their diet was 50 mg. per 100 gm.

TABLE I

*Variation in Magnesium and Water Content of White Rat with Growth and Development*

No. of animals	Age	Average weight	Magnesium			Mean water content	Magnesium
			Range	Mean	$\sigma$		
	wks.	gm.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	per cent	mg. per 100 gm. H <sub>2</sub> O
Composite sample of fetuses	10 days			17.9			
	19 "			19.0			
12	0	5	21.0-22.9	21.7	0.6	88	24.7
8	1	15	22.7-27.0	25.2	1.3	78	32.3
8	2	25	27.5-31.5	29.3	1.6	78	37.8
8	3	38	33.5-44.5	36.9	3.2	70	52.8
5	4	58	36.7-44.2	39.2	2.8	69	56.6
4	6	88.5	37.4-40.7	39.3	1.2	67	58.6
9	10-11	116.5	37.5-47.5	40.4	3.2	66	61.2
5	14	175	30.0-36.7	32.7	2.5	62	52.8
5	18	195	30.0-36.8	33.7	2.5	61.5	54.5
3	18*	305	31.8-35.8	33.2			

\* Rats fed stock colony diet.

of dry food. To rule out the possibility that this synthetic diet produced rats of abnormal magnesium content, a check series of analyses was carried out on rats maintained on the stock colony diet. The results obtained are given in Table I. In the table there are reported the number of animals in each group, their age, average weight, the range, arithmetical mean, and the standard deviation from the mean of the magnesium content of each group, the mean percentage water content, and the calculated magnesium content per 100 gm. of water.

## DISCUSSION

Examination of the data in Table I shows that the magnesium level of the rat is least in the fetus and newly born animal and that subsequently it increases in percentage content during lactation to a maximum value of about 40 mg. per cent at 4 weeks of age. This maximum level persists through the age of 11 weeks, after which a reduction sets in, which, at 14 and 18 weeks of age, amounts to a decrease of about 20 per cent. The magnesium values of the rats between the ages of 4 and 11 weeks, reported in Table I, are in good agreement with the figures of Buckner and Peter and of Medes and Humphrey, and therefore need not be further considered. In support of the correctness of the magnesium values during the lactation period, the evidence may be cited that they are in good agreement with the published values of Toverud (4) on new born rats, and that they harmonize with the data on the change in the calcium and phosphorus levels of the growing rat which also show a marked increase in amount during lactation.

The comparable results obtained on rats reared on the stock colony diet with those on the synthetic ration support the plausibility of the decline in the magnesium content noted at 14 and 18 weeks of age. This decline may possibly be related to the accumulation of adipose tissue by the adult animals.

No sex differences of a significant nature were noted at any period in the analytical series given in Table I. For this reason, the analyses of males and females are grouped together in the table. As an illustration of this, the nine rats of the age group 10 to 11 weeks consisted of five males and four females. The range of magnesium values on these animals was 37.9 to 47.5 mg. per 100 gm. for the males, and 37.5 to 44 for the females. The arithmetical mean in each instance was 40.4 mg. per cent. From these data the reality of a difference in the percentage magnesium content of the two sexes at comparable ages seems illusory.

While the magnesium level is increasing, the water content is progressively decreasing during the life of the rat. In Table I the decrease is from the value of 88 per cent at birth to that of 61.5 per cent at 18 weeks of age. The data on water content are in good agreement with the values cited by Donaldson (5). No greater constancy is obtained if the values of body magnesium are



calculated on the basis of the percentage water content of the rat rather than on the basis of per cent of total body weight.

#### SUMMARY

1. Determination of the magnesium content of normal rats shows that a rapid increase occurs during the lactation period up to 4 weeks of age, followed by a constant level which is maintained through 11 weeks of age. Subsequently, from 14 weeks of age on, there appears a reduction of about 20 per cent.

2. No significant difference was noted in the magnesium level of the two sexes.

3. The water content of the normal rat progressively decreases during its life span.

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## THE DIASTATIC ACTIVITY OF RAT SALIVA

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It is well known that not all warm blooded animals possess a salivary amylase. Mendel and Underhill (1) found that this enzyme is absent in the saliva of the dog and the cat. Schwarz and Rasp (2) added to the list of animals whose saliva contained no amylase the cat, sheep, and goat. The enzyme was present, however, in the pig, mouse, and rat saliva. These workers stated that the quantitative determination of salivary amylase in the mouse and rat was not possible because of the difficulty in securing adequate amounts of saliva. Further studies by Schwarz and Steinmetzer (3) demonstrated the absence of diastatic activity in the saliva of the dog, horse, and steer. Contrary to the findings of most workers, Chrzascz and Schechtlowna (4) found the diastase activity of horse saliva to be about 2 to 4 times as great as that of cattle saliva. These workers also observed that sodium chloride promoted diastase activity. Jung (5) found a weak amylolytic power for dog saliva with boiled starch as the substrate.

The similarity of the metabolic processes of an experimental animal and the human determines to some degree the extent to which the results obtained from a laboratory investigation may be considered representative of what occurs in man. The important experimental rôle of the white rat in the development of the knowledge of nutrition adds significance to investigations of the normal metabolic processes of this species. The present investigation has been designed to determine quantitatively the diastatic activity of rat saliva. It has been possible to demonstrate that the saliva of this rodent contains a digestive ferment which will act upon a starch suspension to produce reducing sugars in a

manner quite analogous to that by which human saliva acts upon the same substrate. It had been shown by Schwarz and Rasp (2) that achroodextrin was produced in a dilute and specially prepared suspension of starch incubated with rat saliva. The evidence of these workers was based upon the disappearance of the blue color given by starch in the presence of iodine. In the present investigation, the formation of reducing sugars has been definitely established by means of Fehling's test. A quantitative estimation of the amount of reducing sugar in terms of the amount of cuprous oxide produced was made with the aid of a specially devised photoelectric colorimetric measurement. By the use of this method, it has been demonstrated that the amount of reducing sugar, as measured by the amount of cuprous oxide precipitated, increases as the time of incubation of starch with saliva is extended.

#### EXPERIMENTAL

*Collection of Saliva*—Rat saliva was collected by allowing a rat, from which food and water had been withheld for approximately 17 hours, to attack a block of sterile rubber sponge. It was thought that this method of collecting saliva would simulate the conditions which exist in a rat ingesting food. The sponge was approximately  $5 \times 1 \times 1$  cm. The sponge was moistened with distilled water and was placed on a clean, dry glass plate in the bottom of the rat cage. The rat was placed on the glass floor with the sponge. After the animal had chewed at the rubber sponge for 1 to 5 minutes, the latter was transferred to a tube containing 10 cc. of 2 per cent starch solution, 4 cc. of phosphate buffer of pH 6.8, 4 cc. of 0.5 per cent NaCl solution, and 2 cc. of toluene, the latter being used as a preservative. The tubes were stoppered with a rubber stopper through which a finely bored glass tube was inserted to allow the pressure on the inside of the tube to be maintained at atmospheric pressure, but at the same time to prevent undue evaporation. The tubes were placed in an incubator which was maintained at a constant temperature of  $38^{\circ}$ .

*Precipitation of Cuprous Oxide*—1 cc. samples of the incubation mixture were withdrawn at intervals. The samples were placed in sterile tubes and heated in a water bath at boiling temperature for 1 minute in order to destroy any enzyme present. At the end

of the incubation time all the samples were filtered to remove the unchanged starch. To each filtrate were added 1.5 cc. of a stock Fehling's solution. The tubes were then placed in a boiling bath for 5 minutes, then removed, and cooled to room temperature. Each individual specimen was filtered and the precipitate of  $\text{Cu}_2\text{O}$  washed with water warmed to  $60^\circ$ . The samples of an entire single incubation were simultaneously treated with Fehling's solution to avoid errors due to possible variations in heating time.

*Determination of Cuprous Oxide*—The small quantities of  $\text{Cu}_2\text{O}$  formed necessitated the use of a sensitive method for the quantitative determination of the amounts of  $\text{Cu}_2\text{O}$  formed. A photometric method was devised for this purpose. The  $\text{Cu}_2\text{O}$  on the filter was dissolved by 1 cc. of concentrated  $\text{HNO}_3$ , added drop by drop from a pipette; the filter was washed once with 1 cc. of distilled water. The filtrate which now contained the copper oxide of each specimen was neutralized with concentrated  $\text{NH}_4\text{OH}$ , added drop by drop to the appearance of a faint blue tint. The solution became turbid due to the presence of impurities; it was filtered and the filter paper washed with 1 cc. of distilled water. The acidity was adjusted by the following procedure. To the clear filtrate 10 per cent  $\text{HNO}_3$  was added, drop by drop, until the solution was acid, as evidenced by the disappearance of the blue tint; 3 M  $\text{NH}_4\text{OH}$  was added to the first reappearance of blue tint; then 1:20  $\text{HNO}_3$  was added until the blue color disappeared. This procedure was followed to minimize the final volume.

Each sample was diluted to exactly 6 cc. The solution from each individual specimen at this time was transferred to a flat bottomed, cylindrical container and its light transmission determined with the aid of a photoelectric colorimeter. The galvanometer reading at this stage is designated  $I_0$  for the specimen in question. (The construction of the colorimeter is indicated below.) After the intensity had been determined in this manner, 5 cc. of a sodium citrate-sodium carbonate mixture<sup>1</sup> were added to produce a fixed alkalinity and a protective medium in which a stable complex copper ion would be formed. Owing to the

<sup>1</sup> The sodium citrate-sodium carbonate solution contained 173 gm. of sodium citrate and 100 gm. of anhydrous sodium carbonate dissolved in 600 cc. of water with the aid of heat. The solution was cooled and diluted to 850 cc.

presence of the ammonium nitrate from neutralization, the appearance of color is to be attributed to the copper-ammonia complex. The intensity of light transmitted through the solution immediately after the addition of sodium citrate-sodium carbonate solution was then determined by the galvanometer reading. This galvanometer reading was designated as  $I$  for the specimen in question.

The logarithm of the ratio of the first and second reading, that is  $\log I_0/I$ , for each specimen was a measure in arbitrary units of the quantity of copper color produced within the given specimen and, therefore, a measure of the  $\text{Cu}_2\text{O}$ . To standardize this read-

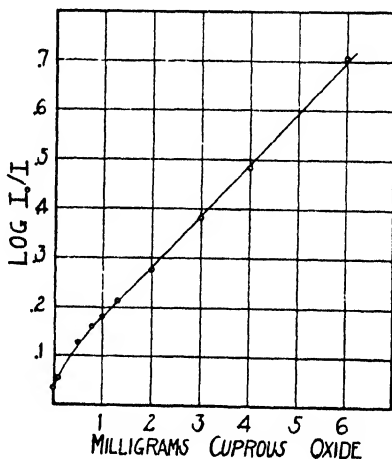


FIG. 1. Calibration curve for the determination of cuprous oxide

ing, weighed quantities of chemically pure  $\text{Cu}_2\text{O}$  were treated in a manner exactly similar to the specimens and the logarithm of initial intensity to final intensity for these specimens obtained. A curve was drawn for these standard copper solutions with the mg. of  $\text{Cu}_2\text{O}$  as abscissa and  $\log I_0/I$  as ordinate. This curve is shown in Fig. 1. To obtain the mg. of  $\text{Cu}_2\text{O}$  in the unknown specimen, it was necessary only to find on the curve the abscissa for the particular value of  $\log I_0/I$  of a specimen.

The quantity of reducing sugar produced in an incubation of the saliva of the rat with the starch suspension described is indicated in Table I. This table represents the values of  $\text{Cu}_2\text{O}$  obtained from a group of five rats with the values for the starch

control incubated under the same conditions. The time which each rat chewed the sponge was variable and is indicated in Table I. Owing to the fact that the amount of rat saliva obtained is variable, and inasmuch as enzymatic potency of collected specimens may differ, only the increase in quantity of reducing sugars with time is significant.

*Theory of Method*—The theory which is the basis of the method of determination of the  $\text{Cu}_2\text{O}$  formed is of interest because of its possible usefulness in applications of the photoelectric method to other biochemical problems. From Beer's (6) law it follows that

TABLE I

*$\text{Cu}_2\text{O}$  Produced by Incubation of Rat Saliva with Starch Solution\**

Incubation time	$\text{Cu}_2\text{O}$ produced					
	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Blank, starch alone
hrs.	mg.	mg.	mg.	mg.	mg.	mg.
0	0	0	0	0	0	0
4	0 05	0 50	0 35	0 10	0 40	0 10
14		0 85	0 30	0 25		0 20
25	0 45	2 0		0 40	1 35	0 30
33 5		2 15	0 50	0 50	1 80	0 35
51 5	0 90	2 25	0 55	0 60	1 70	0 55
96	1 00	2 30	0 50	0 60	3 0	0 40
124	1 40	2 25	0 60	1 40	2 9	0 40
Time of chewing sponge, min. . . .	2 75	3 25	1 0	5 0	5 0	

\* As described in the text.

the change in intensity,  $dI$ , which a beam of light of intensity,  $I$ , undergoes when passing through a layer,  $dX$ , of material whose concentration is  $C$ , is given by the relation

$$dI/I = -KCdX$$

This equation assumes that each unit quantity of material present to absorb light absorbs the same amount of light whether the material is present in a dilute or concentrated condition. Upon integration the equation

$$\log I_0/I = K_1CX$$

is obtained, in which  $I_0$  represents the initial intensity of the light and  $I$  the intensity after passing through a solution of concentration  $C$  and length  $X$ . Inasmuch as the intensity of light transmitted to the photoelectric cell can be considered as proportional to the galvanometer deflections, the galvanometer deflections themselves may be substituted for  $I_0$  and  $I$  respectively, since the ratio would be independent of the units chosen for the light intensity. If the length of path through the liquid,  $X$ , is constant, one can see that the concentration,  $C$ , from the above equations will be given by the relationship

$$C = Q \log I_0/I$$

where  $Q$  is a constant. In the actual method given,  $I_0$  is taken for a height which corresponds to 6 cc. of solution, whereas  $I$  is given for 11 cc. The ratio is taken in this way to correct experimentally the effect of any turbidity in the specimen, since the additional liquid added (citrate-carbonate mixture) will always be equivalent to a fixed value of additional absorption. The method of obtaining the exact equivalent concentration of  $\text{Cu}_2\text{O}$  from the graph of values of the  $\log I_0/I$  against mg. of  $\text{Cu}_2\text{O}$  corrects the use of the equation for the different heights of the solution. It will be observed that 0 mg. of  $\text{Cu}_2\text{O}$  will correspond to a definite value of  $\log I_0/I$ . The method of obtaining the amount of  $\text{Cu}_2\text{O}$  from the graph is a device which also compensates for the possible non-validity of Beer's law and the possibility that the galvanometer deflections may not be exactly directly proportional to the intensity of light. The copper calibration curve will vary with the relative amounts of initial solution and sodium citrate-sodium carbonate mixture, with the light filter used, with the intensity-sensitivity curve of the photoelectric cell used, and with the wave-length sensitivity curve of the photoelectric cell employed.

*Photoelectric Colorimeter*—The photoelectric colorimeter was constructed by placing a light source consisting of a 40 watt filament bulb inside a metal housing and allowing this light to fall upon a long focus lens, the lens being placed so that the distance from light to lens was equal to the focal length of the lens. The parallel light thus obtained passed through the solution contained in the flat bottomed, cylindrical colorimeter tube. The tube

rested upon the edges of a hole drilled through a horizontal ledge and the light fell upon the sensitive surface of a Weston photronic cell. The terminals of the photronic cell were connected to a d'Arsonval galvanometer, the deflection of which was read with a telescope and scale. For a given calibration curve the series resistance is fixed. By changing the value of the resistance, however, it is possible to obtain calibration curves for concentrations both smaller and larger than those reported in this paper. The voltage of the electrical supply to the 40 watt bulb was held constant by means of a voltage-regulating transformer.

Because the color of the copper ion under the conditions of photometry is green-blue, indicating a preferential absorption in the yellow and red end of the spectrum, a glass filter, which absorbed in the blue and green portions of the spectrum, was used to filter the light which fell upon the solution and the photronic cell. This was done to increase the relative sensitivity of the photronic cell to the color absorbed by the copper complex ion formed. The filter used was an HR, "red shade yellow," Corning glass filter of 2.98 mm. thickness.

#### SUMMARY

By a method which makes use of the common Fehling's test for reducing sugars, results have been obtained which indicate that the saliva of the rat contains an enzyme capable of acting upon starch to form reducing sugars. The amount of reducing sugar produced has been measured quantitatively in terms of cuprous oxide by means of a photoelectric method.

The authors wish to express their thanks to Dr. Byron E. Cohn of the Department of Physics of the University of Denver for aid given in the development of the method used for the photoelectric determination of the cuprous oxide.

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## A COLORIMETRIC METHOD FOR THE DETERMINATION OF INORGANIC SULFATE IN SERUM AND URINE\*

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The study of serum sulfate has been handicapped by uncertainty concerning the accuracy and dependability of available micromethods for determination of inorganic sulfate. While a variety of procedures have been described, the results obtained differ, depending on the method employed. Thus Hoffman and Cardon (5) have tabulated values found by the application of various methods to analysis of normal blood serum. The lack of agreement is impressive and Hoffman and Cardon justly conclude that practically all procedures that have been advocated give incorrect results. These authors propose a modified oxidimetric, benzidine precipitation technique that yields for most normal specimens of serum less than 1 mg. per cent of inorganic sulfur. We have for some time engaged in an investigation of serum inorganic sulfate and sulfate clearance with the aid of a revised colorimetric benzidine method. The average normal concentration that we have found is roughly one-third higher than the corresponding average published by Hoffman and Cardon. Careful examination of our procedure fails to reveal a source of error of this magnitude. Criteria of purity for the benzidine sulfate precipitate mentioned by Hoffman and Cardon appear to be fulfilled in our method. Solutions containing known concentrations of inorganic sulfate are correctly analyzed and sulfate added to serum can be recovered with reasonable accuracy. Furthermore, comparison of the colorimetric method with a standard macrogravimetric method showed close agreement. We are

\* The technique was described and demonstrated at the May, 1934, meeting of the Physiological Society of Philadelphia.

obliged to conclude, as a consequence, that the method proposed by Hoffman and Cardon yields somewhat low results. It is our belief that the average normal serum inorganic sulfur concentration is slightly over 1 mg. per cent.

The improved colorimetric procedure for the determination of inorganic sulfate described in the present paper avoids objectionable features of several of the older methods that cause such methods to give incorrect results. The modified method is an outgrowth of unsuccessful attempts to use the colorimetric method of Hubbard (6), as adapted by Wakefield (10), described by Peters and Van Slyke (8). The principal difficulty encountered was the feebleness and instability of the color given by benzidine, when treated with ferric chloride and hydrogen peroxide. A search was made, consequently, for other reagents that might give a stronger and more stable color. Of the substances tested, sodium  $\beta$ -naphthoquinone-4-sulfonate was the most promising. It has been employed by Folin (4) for the determination of amino acids, and is known to give intense red colors with aniline and similar compounds. With benzidine in alkaline solution, sodium  $\beta$ -naphthoquinone-4-sulfonate develops a red-brown color which changes to red on addition of acetone. This color is stable and well adapted to colorimetry. Furthermore, the yellow color of the excess of reagent is almost entirely discharged by the acetone.

Another innovation, so far as sulfate determination is concerned, is the use of uranium acetate as a precipitant of protein. Inorganic phosphorus is removed by this reagent simultaneously with protein, hence error due to precipitation of benzidine by phosphate and the consequent need for removing phosphate as a separate step in the method are avoided.

### *Procedure*

*Reagents*—Distilled water of known purity should be employed.

Uranium acetate solution, 0.80 gm. of uranium acetate in 200 cc. of water.

Benzidine solution. 1 gm. of benzidine is dissolved in 100 cc. of acetone and filtered. The solution is stored in a refrigerator in a brown bottle and discarded when it becomes highly colored.

Glacial acetic acid.

Acetone, 99.5 per cent.

Standard benzidine hydrochloride solution. 0.1606 gm. of purified<sup>1</sup> benzidine hydrochloride is transferred to a 200 cc. volumetric flask, dissolved in water previously warmed to about 50°, cooled, and diluted to volume. The solution should be stored in the cold. 10 cc. are equivalent to 1.0 mg. of sulfur. For a working standard, 10 cc. of this solution are diluted to 100 cc. with water. 1 cc. contains benzidine equivalent to 0.01 mg. of sulfur. The solution should be stored in the cold.

Sodium hydroxide-sodium borate solution. 1 gm. of powdered sodium borate is dissolved in 100 cc. of 0.1 N sodium hydroxide. Preserve in a Pyrex bottle.

Sodium  $\beta$ -naphthoquinone-4-sulfonate solution. 0.15 gm. of a pure preparation is dissolved in 100 cc. of distilled water. The solution will keep about 2 weeks in the cold. Each sample of this reagent should be tested by treating 2 cc. and 4 cc. of the working standard solution of benzidine hydrochloride with the color reagent, borate, water, and acetone as described below. Acceptable preparations do not deviate from the theoretical Beer's law relationship by more than 5 per cent.

*Determination of Sulfate in Serum*—6 cc. of uranium acetate solution are measured into a 15 cc. centrifuge tube and 2 cc. of non-hemolyzed serum are added slowly. After mixing by inverting four times, the mixture is centrifuged for 10 minutes. The clear fluid is transferred to a test-tube by means of a medicine dropper.

4 cc. of the centrifugate are measured into a centrifuge tube (selected so that the tip will retain precipitates). 1 cc. of glacial acetic acid is added. This is followed by 9 cc. of benzidine solution. The tube is capped and placed in ice water for 30 minutes or longer, then centrifuged for 15 minutes at 3000 R.P.M. The

<sup>1</sup> In the purification of benzidine hydrochloride 5 gm. of benzidine hydrochloride are dissolved in 200 cc. of 5 per cent hydrochloric acid by warming to about 50°. Any insoluble residue is filtered. 20 cc. of concentrated hydrochloric acid are added with continuous stirring. The solution is cooled in ice water for about 30 minutes, when the crystals that have formed are collected on a Buchner funnel. The material is washed with cold diluted hydrochloric acid (15 cc. of concentrated acid to 100 cc. of water). After removing the hydrochloric acid by a vacuum, the crystals are washed with two 25 cc. portions of cold ethyl alcohol and four portions of ether. After all traces of ether are removed, the dry crystals are transferred to a brown bottle.

supernatant fluid is decanted and discarded. The tube is permitted to drain in an inverted position for 3 minutes. 14 cc. of acetone are added. The precipitate is suspended in the acetone, then again centrifuged for 15 minutes at high speed. The acetone is decanted and the tube allowed to drain 5 minutes. After the mouth of the tube has been wiped, 1 cc. of the borate solution is added and the precipitate is dissolved by stirring. (The tube may be placed in warm water at 60° if solution is slow.) Finally 10 cc. of water and 1 cc. of the color reagent are added. The solutions are mixed and allowed to stand 5 minutes, then 2 cc. of acetone are added. At the same time, two standards are prepared by measuring 2 and 5 cc. of benzidine hydrochloride solution into two test-tubes. 1 cc. of borate solution is added to each, together with 8 cc. and 5 cc. of water respectively. 1 cc. of color reagent is added and the development of color carried out as described. The unknown solutions are compared with standards in the colorimeter. Calculation is made by the usual formula.

*Determination of Sulfate in Urine*—To 1 cc. of urine are added 4 cc. of 0.4 per cent uranium acetate solution. Depending on the volume of urine secreted, dilution with water is carried out as follows: for urine volumes less than 50 cc. per hour dilute to 20 volumes; between 50 and 100 cc. per hour, dilute to 10 volumes; between 100 and 200 cc. per hour, dilute to 5 volumes; between 200 and 300 cc. per hour, dilute to 2 volumes; over 300 cc. per hour, omit dilution. After mixing, the precipitate of phosphate and protein is removed by filtration. 1 cc. of the filtrate is analyzed as described for the protein-free centrifugate of plasma. Calculation is made as indicated by taking into account the dilution employed.

#### EXPERIMENTAL

Uranium acetate was found to be the most satisfactory of numerous agents tested for removal of proteins preliminary to sulfate precipitation. Phosphate is removed with the protein. While normal concentrations of phosphate in serum exert no appreciable influence on the results of inorganic sulfate determination by the technique described, phosphate in high concentration may be precipitated by benzidine and so cause high results.

Also in analysis of urine for inorganic sulfate, removal of phosphate is a necessary preliminary step. Uranium acetate is convenient and highly effective for this purpose. We have failed to find any effect of chloride in concentrations encountered in serum or urine. Hoffman and Cardon imply that chloride may cause high results. It is to be noted that these authors introduce appreciable amounts of chloride with their benzidine reagent.

The choice of a protein precipitant may definitely influence the results of sulfate determinations. Higher values are obtained when trichloroacetic acid is employed for removal of proteins, as compared with figures found when uranium acetate is used. Sixteen determinations of inorganic sulfate in samples of horse serum averaged 2.82 mg. per cent as sulfur when protein was precipitated by uranium acetate. The same samples yielded an average of 3.56 mg. per cent of sulfur when trichloroacetic acid (purified sulfate-free) was employed, as described by Hubbard for removal of protein. Deproteinization by heat in the presence of 1 per cent acetic acid gave even higher results, ranging 10 per cent above the trichloroacetic acid figures. On the other hand, sulfate analyses following removal of protein by the reagents employed by Hoffman and Cardon (ferric chloride-ammonium hydroxide-ammonium acetate) agree with the values found by the use of the uranium acetate precipitant.

The increase of 25 to 35 per cent in inorganic sulfur caused by the use of acid precipitating agents apparently represents liberation of sulfate from a constituent of serum. An analogous increase in inorganic phosphate when serum stands in the presence of trichloroacetic acid has been described by Kay (7), who attributes the change to decomposition of phospholipid. However the rise in inorganic sulfate differs in that it appears to occur at once on addition of acid. The readiness with which sulfate is liberated suggests that the sulfate so obtained is associated or combined with protein, since there is no similar change on acidification of uranium filtrates. The presence of phosphate in the filtrates when acid precipitants are used apparently does not explain this discrepancy. Phosphate added to uranium acetate filtrates in concentrations equal to or exceeding those of the serum employed fails to influence the results appreciably. It is evident that the

use of trichloroacetic acid under these circumstances explains the higher values found by many authors. Liberation of sulfate from protein by bromine or by fuming nitric acid has been described by Blumenthal and Clarke (1).

Quantitative precipitation of sulfate by benzidine requires an acid reaction, although acidity must not be excessive. Acetic acid, added as directed, fulfils requirements satisfactorily. The precipitate is white and crystalline and free from discoloration. On standing in ice water, the crystals become aggregated into clumps. Unless aggregation occurs, loss of finely divided crystalline material with the supernatant fluid becomes a factor. For washing the precipitated benzidine sulfate, undiluted acetone is employed. Hoffman and Cardon found it necessary in their technique to use 90 per cent acetone for complete removal of benzidine hydrochloride. Benzidine sulfate is far from insoluble in this concentration of acetone. We have found that a single washing of the precipitated benzidine sulfate with 90 per cent acetone resulted in losses of 20 to 50 per cent, depending on concentration of sulfate present, even when contact between solvent and precipitate was minimal. Although Hoffman and Cardon by the use of special tubes attempt to avoid loss due to solubility, it is doubtful whether this measure is wholly effective, and we believe that the lower values obtained by these authors are explained in part at least by solution of benzidine sulfate during this operation. The fact that these authors obtain satisfactory recovery of added sulfate cannot be accepted as proof that such loss is avoided or that the results of determinations are correct. Quantities of benzidine sulfate removed in the wash solution would be quite constant and independent of the amount of precipitate under fixed conditions; consequently, recoveries might be quantitative. Hoffman and Cardon do not provide a comparison of their procedure with a standard method, although data are presented showing close agreement with what we conclude is a macromodification of the method they describe. Obviously, errors of the type in question would not be detected by such a comparison.

The color reaction between benzidine and  $\beta$ -naphthoquinone-4-sulfonate is well adapted to colorimetry. However, pure preparations of the color reagent are essential. Several samples that

have been purchased were not suitable for use. It is therefore necessary to test each preparation as described. When pure preparations are employed, the intensity of the color formed deviates only slightly from the expected color intensity for any given concentration over a wide range of concentrations. Such deviations are negligible when concentrations vary within 50 per cent of the concentration of the standard. When the difference in concentration approaches 100 per cent, results are low by about 5 per cent. Color contributed by the necessary excess of reagent is discharged by addition of acetone preceding color-

TABLE I

*Recovery of Sulfate Added to Horse Serum*

Potassium sulfate containing 0.032 mg. of sulfur was mixed with horse serum containing 0.0282 mg. of inorganic sulfate sulfur and analyzed at once.

S found	Added S recovered	
mg.	mg.	per cent
0.0590	0.0308	97
0.0563	0.0286	90
0.0574	0.0292	91
0.0544	0.0262	82
0.0557	0.0272	86
0.0594	0.0312	98
0.0577	0.0295	92
0.0575	0.0293	92
0.0591	0.0309	96
Average.....		91.6

imetry. A compensating colorimeter may be used but is not essential if the color reagent is of good quality. A borate buffer for use in conjunction with  $\beta$ -naphthoquinone-4-sulfonate as suggested by Danielson (2) has been adopted. Blank determinations without sulfate have been negative without exception.

Inorganic solutions containing known amounts of sulfate can be analyzed with acceptable precision. Seventeen separate examinations of the concentration of inorganic sulfate in a sample of horse serum showed an average deviation from the mean of 2.2 per cent. The greatest single differences were +5.7 per cent and -2.4 per cent.



Sulfate added to serum can be recovered satisfactorily provided analysis is started at once after mixing the added sulfate with the serum (Table I). When this was done, the average recovery by the method described was 91.6 per cent in nine experiments. However, if analysis was delayed 5 to 10 minutes, recoveries averaged only 77 per cent, while longer delay led to larger losses of 40 to 50 per cent. This effect was observed regardless of the protein precipitant used and similar results were obtained when trichloroacetic acid, alcohol, or heat was employed

TABLE II  
*Analysis of Sulfate of Urine by Colorimetric and Gravimetric Methods*

Specimen No.	Inorganic S excreted during 24 hrs.	
	Colorimetric analysis	Gravimetric analysis
	gm.	gm.
1	0.63	0.62
2	0.62	0.62
3	0.60	0.59
4	0.60	0.60
5	0.95	0.95
6	1.10	1.10
7	0.46	0.47
8	0.39	0.40
9	0.38	0.38
10	0.47	0.46
11	0.40	0.40
12	0.46	0.47
Average.....	0.59	0.59

for removal of protein. These findings suggest that sulfate added to serum becomes firmly bound, and is thus rendered non-precipitable by benzidine. One may surmise that a similarly bound fraction preexists in serum. However, the liberation of sulfate by acid previously described may not be and probably is not related to the disappearance of added sulfate, since the latter occurs also when acid precipitants are employed.<sup>2</sup>

Power, Keith, and Wakefield (9) have reported that adminis-

<sup>2</sup> Conversion of sulfate added to serum to a non-precipitable form has not been mentioned by previous writers on the subject. The experiments presented in Table I were made with horse serum preserved with phenol; however, normal human serum without preservative behaved in a like manner.

tration of acacia intravenously markedly influences the results of serum sulfate determinations by their oxidative method. The results of our colorimetric method are not affected by the presence of acacia, although acacia is precipitated by acetone simultaneously with benzidine sulfate. It is not unlikely that other oxidizable material at times may escape precipitation with protein and yet be thrown down by acetone, thus introducing error if determinations are carried out by an oxidimetric technique.

In Table II are shown the results of determinations of inorganic sulfate of urine by the colorimetric method and also by the Folin (3) gravimetric method. The figures agree, a fact that further supports the accuracy of values found by use of our method. Sulfate added to urine can be recovered quantitatively irrespective of the time of analysis. The procedure described has proved to be convenient for the determination of sulfate clearance.

Serum inorganic sulfate of twenty-three normal individuals has been determined by the method described. The ages of the subjects ranged between 20 and 40 years. The figures so obtained fall within the comparatively narrow limits of 0.95 mg. and 1.16 mg. per cent as sulfur. It is believed that the average, 1.04 ( $\pm 0.049$ ) mg. per cent, represents the normal inorganic sulfur of serum more accurately than the higher or lower average levels that have been reported in the literature.

#### SUMMARY

A convenient colorimetric procedure for the determination of sulfate in serum and urine, based on the color-producing reaction between benzidine sulfate and sodium  $\beta$ -naphthoquinone-4-sulfonate, is described. Phosphate and proteins are removed preliminary to analysis by the use of uranium acetate solution.

The inorganic sulfur of normal human serum, determined by this method, averages 1.04 mg. per 100 cc. The lowest value was 0.95 mg., the highest, 1.16 mg. per 100 cc. Additional sulfate is liberated when serum is treated with trichloroacetic acid for removal of proteins. Sulfate added to serum is rendered partially non-precipitable by benzidine.

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## THE IRON CONTENT AND OXYGEN CAPACITY OF BLOOD

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In the course of studies on the acid-base metabolism of different organs in dogs, it was necessary to know the hemoglobin concentration of arterial and venous blood with an accuracy of 0.1 mm per liter. With the gasometric method of Van Slyke and Neill (1) it was difficult and at times impossible to obtain checking results on the O<sub>2</sub> capacity of venous dog blood, although with arterial blood duplicate analyses agreed usually to within 0.05 mm. As an expedient under these circumstances, the venous O<sub>2</sub> capacities were calculated from the arterial values, the difference in the water content of the two bloods being used (assuming that hemoglobin varies directly with total solids in arterial and venous blood drawn simultaneously from a given animal), and the directly observed venous capacities were always lower than these calculated values, sometimes by as much as 10 per cent. This difficulty was later traced to the fact that after the blood was drawn, it was kept *at its original tension* over mercury for several hours, until the analyses for O<sub>2</sub> and CO<sub>2</sub> content could be completed, before the blood was aerated for the capacity determinations. Neill and Hastings (2) have shown that methemoglobin formation takes place spontaneously when blood is allowed to stand, and that this occurs more rapidly in *partially reduced blood* than in either fully oxygenated or fully reduced blood. We found that, if venous blood is aerated immediately after drawing from an animal, the O<sub>2</sub> capacity values show excellent agreement among duplicates, and also agree with the values for arterial blood having the same water content and drawn simultaneously from the same animal. Also the capacity values on such immediately aerated bloods do

not change when the blood is allowed to stand for 48 hours at refrigerator temperature. Our difficulties with the O<sub>2</sub> capacity determinations on venous blood were therefore due to a failure to realize the importance of *prompt* aeration of the blood after drawing from an animal.

These difficulties with the gasometric oxygen capacity determination stimulated us to seek another method by which the accuracy of the gasometric method could be checked. This paper presents the details of the determination of iron in blood of the required accuracy and shows that the two methods agree to within 1 per cent, the limit of error of the iron method.

A review of the literature of the past 20 years shows many studies (3, 4) on the iron content of blood, but in none of these is the accuracy as great as 1 per cent. Probably the most accurate of these is the work of Blackwood and Stirling (4), in which iron is determined iodometrically in 2 cc. samples of blood. They worked out a set of conditions of acidity and concentration of KI which gave iron values within 2 per cent of the theoretical amount present in standard FeCl<sub>3</sub> solution, when 0.005 Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> was used in the titration.

As part of our effort to develop a method for iron in blood which would be accurate to at least 1 per cent, the method was tried of oxidizing the blood with H<sub>2</sub>SO<sub>4</sub> and HNO<sub>3</sub>, reducing the iron with zinc, and titrating the resulting ferrous sulfate with KMnO<sub>4</sub>. We found that it was quite possible to determine iron in standard ferric sulfate solutions with the required accuracy by this method, but that in titrating the iron solutions obtained from oxidized blood, something was present which interfered with the definiteness of the KMnO<sub>4</sub> end-point, so that an accuracy greater than 2 or 3 per cent was impossible. Even though the iron was precipitated as Fe(OH)<sub>3</sub> and redissolved in H<sub>2</sub>SO<sub>4</sub> before the reduction and titration, this difficulty could not be avoided.

The method which we finally found to give the required accuracy was an iodometric method which differs from that reported by Blackwood and Stirling (4) in several details, involving particularly control of the concentration of salt as well as of acid during the thiosulfate titration, and a special procedure for the standardization of the thiosulfate solution.

*An Iodometric Method for the Determination of Iron in Blood*

In general the method is as follows: The blood is oxidized with concentrated  $\text{H}_2\text{SO}_4$ , concentrated  $\text{HNO}_3$ , and 30 per cent  $\text{H}_2\text{O}_2$ ; the iron is precipitated as ferric hydroxide by the addition of  $\text{NH}_4\text{OH}$ ; the precipitate is collected on a filter, washed free from sulfate, and redissolved in  $\text{HCl}$ ; the acid solution is partially neutralized with  $\text{NaOH}$ ,  $\text{KI}$  is added, and the liberated iodine is titrated with  $\text{Na}_2\text{S}_2\text{O}_3$ .

*Reagents—*

1. 1.2 N  $\text{HCl}$  (approximate). Dilute 100 cc. of concentrated  $\text{HCl}$  to 1 liter with water.

2. 0.6 N  $\text{HCl}$  (approximate). Dilute 1 volume of the 1.2 N  $\text{HCl}$  with 1 volume of water.

3. 6 N  $\text{NaOH}$  (approximate). Dissolve 240 gm. of  $\text{NaOH}$  in 1 liter of solution.

4. 1 per cent soluble starch. Dissolve 1 gm. of soluble starch in 100 cc. of boiling water and heat for 1 or 2 minutes until the starch is dissolved. Merck's soluble starch (according to Lintner) does not give a red or purple color with  $\text{I}_2$  (characteristic of dextrin) and is therefore suitable. This solution should not be used after more than 12 hours standing.

5. 20 per cent  $\text{KI}$ . Dissolve 20 gm. of  $\text{KI}$  in 100 cc. of solution. This solution decomposes appreciably in the course of 10 or 12 hours.

6. 0.1 N  $\text{Na}_2\text{S}_2\text{O}_3$  (approximate). Dissolve 24.82 gm. of  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  in water and make up to 1 liter.

7. 0.0125 N  $\text{Na}_2\text{S}_2\text{O}_3$ . Dilute 25 cc. of the 0.1 N stock solution of  $\text{Na}_2\text{S}_2\text{O}_3$  to 200 cc. This solution is put in the reservoir of a 5 cc. Koch microburette (5) graduated to 0.01 cc., the tip of which is of such a size that 1 drop equals 0.01 cc.

8. Standard 0.01 N  $\text{FeCl}_3$  solution. Dissolve 559.2 mg. of an analyzed grade of iron wire (99.89 per cent pure) in 5 cc. of hot concentrated  $\text{HCl}$  and 20 cc. of water in a standardized 1 liter volumetric flask. When the wire is completely dissolved, gradually add 6 N  $\text{NaOH}$  with vigorous shaking, during which time the green gelatinous precipitate of  $\text{Fe}(\text{OH})_2$  which forms locally is oxidized by the air to ferric ion. The ferric ion remains in solution as  $\text{FeCl}_3$  until enough  $\text{NaOH}$  has been added to neutralize

the acid present, at which time  $\text{Fe}(\text{OH})_3$  precipitates. Then add 1 N NaOH a few drops at a time.  $\text{Fe}(\text{OH})_2$  is precipitated locally and is oxidized to  $\text{Fe}(\text{OH})_3$  by shaking the flask vigorously until the dark brown color disappears, leaving only the red  $\text{Fe}(\text{OH})_3$ . Continue to add N NaOH in this manner until there is no further darkening of the solution at the point of addition of the alkali due to the formation of  $\text{Fe}(\text{OH})_2$ . Do not add more than 10 drops of NaOH at a time, since it is difficult to oxidize and to redissolve the precipitate if large amounts of  $\text{Fe}(\text{OH})_2$  are formed at one time. Let the suspension of  $\text{Fe}(\text{OH})_3$  stand about 1 hour exposed to air. Then add enough concentrated HCl to dissolve completely the  $\text{Fe}(\text{OH})_3$  and any small traces of  $\text{Fe}(\text{OH})_2$  which may remain unoxidized. Add just enough 6 N NaOH to cause a permanent although not complete precipitation of  $\text{Fe}(\text{OH})_3$ , and shake the solution with air a final time to insure complete oxidation of ferrous ion. Then add 50 cc. of concentrated HCl, making the final solution 0.6 N with respect to HCl and make up to volume with water.

#### *Procedure*

*Oxidation of Blood*—A 3 or 5 cc. sample of blood, measured with an accuracy of 1 part in 200, is introduced into a 300 cc. Kjeldahl flask, followed by 1 cc. of concentrated  $\text{H}_2\text{SO}_4$ .

The blood is oxidized by adding four to six successive 0.5 cc. portions of concentrated  $\text{HNO}_3$  and heating on a rack over a microburner after each addition. In the early stages of the oxidation, care must be taken not to heat so long between additions of  $\text{HNO}_3$  that the charring residue dries where the flame strikes the glass, in order to avoid cracking of the flask. When the charred semisolid residue begins to spatter, another addition of  $\text{HNO}_3$  should be made. Later, when the residue no longer contains solid, one heats until white  $\text{SO}_3$  fumes appear before the next addition of  $\text{HNO}_3$ . When the acid solution appears clear and the only color is that due to the pale yellow solid ferric sulfate, a final addition of  $\text{HNO}_3$  is made in such a way that it rinses down any particles which may be adhering to the neck of the flask, and the mixture is heated for about 1 hour until the brown fumes have entirely disappeared, and only white  $\text{SO}_3$  fumes remain. Four 0.25 cc. portions of 30 per cent  $\text{H}_2\text{O}_2$  are added drop by drop

and the mixture heated to the appearance of white fumes after each addition.

The anhydrous solution is cooled, 15 to 20 cc. of distilled water are added, and the solution evaporated over a Bunsen burner until white fumes appear. This evaporation is done to insure the removal of the last traces of  $\text{HNO}_3$ . After cooling, about 30 cc. of water are added and the solution is boiled for about 15 minutes to insure complete resolution of the ferric sulfate. When the ferric sulfate is dissolved, the flask is cooled and 1 drop (0.10 to 0.15 cc.) of 0.01 N  $\text{KMnO}_4$  is added to the solution. If the pink color remains, the oxidation is complete; if not, the solution is evaporated to white fumes and another 0.5 cc. portion of  $\text{HNO}_3$  added and the solution heated to the disappearance of brown fumes, when water is again added, the solution evaporated to white fumes to insure the removal of  $\text{HNO}_3$ , and then another 30 cc. portion of water is added, and the mixture heated to dissolve the ferric sulfate. The cooled solution is again tested with  $\text{KMnO}_4$ .

*Preparation of  $\text{FeCl}_3$  Solution for Titration*—When the solution is completely oxidized, as evidenced by the permanent  $\text{KMnO}_4$  color, 10 cc. of  $\text{NH}_4\text{OH}$  (specific gravity 0.90) are added to the solution in the Kjeldahl flask, which cause the formation of colloidal  $\text{Fe}(\text{OH})_3$ , as shown by a brown cloudiness. The flask is suspended over a hot-plate for 15 or 20 minutes, during which time the cloudiness disappears, the solution becomes clear and colorless, and a red-brown gelatinous precipitate of  $\text{Fe}(\text{OH})_3$  settles out. The precipitate is then collected by filtering the hot ammoniacal solution through a 7 cm. hardened filter paper on a 4 to 5 cm. funnel and is washed about six times with 10 cc. portions of hot ammonia water (1 cc. of concentrated ammonia in 1 liter of water) until a drop of the filtrate no longer shows a positive test for sulfate with 5 per cent  $\text{BaCl}_2$ . The funnel is then transferred to another 125 cc. Erlenmeyer flask.

The Kjeldahl flask is rinsed with 10 cc. of the 1.2 N  $\text{HCl}$  (accurately measured in a volumetric pipette) and this acid is then poured onto the filter paper containing the  $\text{Fe}(\text{OH})_3$  precipitate. A second 10 cc. portion of 1.2 N  $\text{HCl}$  is added in a similar way, first to the Kjeldahl flask and then to the filter paper. This is followed by 10 cc. of 0.6 N  $\text{HCl}$ . The filter paper is allowed to drain completely between each two additions of acid so as to in-



sure complete removal of the  $\text{FeCl}_3$  solution from the filter paper. A second and a third 10 cc. portion of 0.6 N HCl are poured directly on the filter paper from the pipette. The filtrate containing an amount of HCl equivalent to 39 cc. of N HCl is then partially neutralized by the addition of 6 cc. of the 6 N NaOH, leaving approximately 3 cc. of N acid in the 56 cc. of  $\text{FeCl}_3$  solution. Although the normality of the HCl and NaOH are only approximately controlled, the volumes used are exactly measured so that all the individuals in a given series of iron determinations have exactly the same acidity and salt content.

*Titration with  $\text{Na}_2\text{S}_2\text{O}_3$* —5 cc. of the 20 per cent KI are added to the  $\text{FeCl}_3$  solution and the mixture is allowed to stand exactly 2 minutes. Then the 0.012 N  $\text{Na}_2\text{S}_2\text{O}_3$  is added from the Koch burette until the brown iodine color has nearly disappeared. 2 drops of the 1 per cent starch solution are added and the mixture turns blue. More of the  $\text{Na}_2\text{S}_2\text{O}_3$  is now added until the solution is colorless. The solution is allowed to stand 5 minutes, during which time the blue color usually returns. A final addition of 1 to 10 drops of  $\text{Na}_2\text{S}_2\text{O}_3$  brings the solution to a permanent end-point.

Blank determinations are run in exactly the same way on reagents alone. The value of the blank, about 0.05 cc. of 0.01 N thiosulfate, is due almost exclusively to the reagents used in the oxidation of the blood, since a partial blank, run on the reagents used in the precipitation, filtration, and titration, is practically zero.

*Standardization of  $\text{Na}_2\text{S}_2\text{O}_3$* —1, 3, and 5 cc. samples of the 0.01 N standard  $\text{FeCl}_3$  solution are measured with Van Slyke-Ostwald pipettes in 50 cc. Erlenmeyer flasks, the  $\text{Fe}(\text{OH})_3$  is precipitated with  $\text{NH}_4\text{OH}$ , the precipitate collected on a filter, washed with hot ammonia water, and then dissolved in exactly 20 cc. of the 1.2 N HCl and exactly 30 cc. of the 0.6 N HCl as described in the procedure given above. This solution is partially neutralized with exactly 6 cc. of 6 N NaOH and the titration with  $\text{Na}_2\text{S}_2\text{O}_3$  carried out as previously described.

The amount of  $\text{Na}_2\text{S}_2\text{O}_3$  equivalent to 1 cc. of  $\text{FeCl}_3$  (0.5584 mg. of Fe) varies with the amount of  $\text{FeCl}_3$  present, as shown in Table I. Here it is seen that the volume of  $\text{Na}_2\text{S}_2\text{O}_3$  per cc. of  $\text{FeCl}_3$  decreases as the amount of  $\text{FeCl}_3$  used in the standardization in-

creases from 1 to 10 cc., and the factors on the  $\text{Na}_2\text{S}_2\text{O}_3$  correspondingly increase. Since these factors are different and since there is not a constant difference between the factors determined with the 1, 3, and 5 cc. samples of  $\text{FeCl}_3$ , it is necessary to determine factors on the  $\text{Na}_2\text{S}_2\text{O}_3$  by using different amounts of the standard iron solution which have iron contents approximately equal to those of the blood samples. The factor on the  $\text{Na}_2\text{S}_2\text{O}_3$  is, then,

TABLE I

*Analyses of Standard 0.01 M  $\text{FeCl}_3$ , Showing Variations in the Factor on  $\text{Na}_2\text{S}_2\text{O}_3$  with Different Amounts of Iron*

Date	0.01 M $\text{FeCl}_3$	$\text{Na}_2\text{S}_2\text{O}_3$	$\frac{\text{Cc. Na}_2\text{S}_2\text{O}_3}{\text{Cc. FeCl}_3}$	Factor on $\text{Na}_2\text{S}_2\text{O}_3$
July	cc.	cc.		
9	1	0.805	0.805	1.242
	3	2.408	0.802	1.247
	5	4.010	0.802	1.247
9	1	0.795	0.795	1.258
	3	2.380	0.793	1.261
	5	3.968	0.793	1.261
7	1	0.825	0.825	1.211
	3	2.400	0.800	1.250
	5	3.996	0.799	1.251
	10	7.900	0.790	1.266
1-2	1	0.840	0.840	1.190
	3	2.462	0.820	1.219
	5	4.052	0.810	1.234
1-2	1	0.828	0.828	1.207
	3	2.387	0.799	1.251
	5	3.943	0.788	1.269

defined in terms of the amount of  $\text{FeCl}_3$  which is used in the standardization.

If the iron contents of the various blood samples which are being analyzed all fall within a narrow range of a few per cent of each other, as would occur if one were using the same sized samples of blood of nearly the same composition, then one amount of the standard  $\text{FeCl}_3$  solution would be sufficient. In general, however, it is safer to use at least two different amounts of the standard iron solution because of the difficulty of knowing beforehand just how much iron is present in the blood. In our work, where 3 to

5 cc. of blood containing about 10 mm per liter of iron were taken, 3 and 5 cc. amounts of the standard 0.01 N FeCl<sub>3</sub> solution served well to define the factors on the Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. When the amount of iron in the blood fell between the values of these standards, we interpolated to determine the factor on the Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> to be used in calculating the iron content of any particular sample of blood.

### *Calculation and Data*

Four steps are involved in the calculation: calculation of the factors on the thiosulfate for the standard iron solutions and then, by interpolation, for the blood sample; correction for blank on reagents; calculation of the amount of iron (in cc. of 0.01 N, i.e. 10<sup>-5</sup> equivalent) in the sample used; and from the size of the sample, calculation of the concentration of iron in the blood, in mm per liter. These are herewith illustrated.

*Data*—3 cc. of standard 0.01 M FeCl<sub>3</sub> = 2.462 cc. of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. ∴ the Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> factor = 1.219, i.e. the Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> is 0.01219 N.

5 cc. of standard 0.01 M FeCl<sub>3</sub> = 4.052 cc. of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. ∴ the Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> factor = 1.234.

3 cc. of blood = 3.076 cc. of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>.

Blank on the reagents = 0.045 cc. of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>.

By interpolation, the factor on the thiosulfate for the 3 cc. of blood is

$$1.219 + \frac{(3.076 - 2.462)}{(4.052 - 2.462)} (1.234 - 1.219) = 1.225$$

Correcting the Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> titer on the blood for the blank, we get 3.076 - 0.045 = 3.031 cc. of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, net for 3 cc. of blood.

Then 3.031 × 1.225 = 3.713 cc. of 0.01 M iron in 3 cc. of blood.

Finally  $\frac{3.713}{3} = 1.238 \times 10^{-5}$  equivalents of iron in 1 cc. of blood, which is

12.38 mm of iron per liter of blood.

The accuracy of the method is illustrated by the data in Table II, from an experiment in which determinations were made on mixtures of blood and standard FeCl<sub>3</sub> solutions in addition to those on blood alone. It is seen that in those cases where standard FeCl<sub>3</sub> was added to blood, the equivalents of iron are equal to the sum of the amounts present in each constituent with an accuracy of 0.1 mm per liter.

TABLE II

*Analyses of Blood, Standard FeCl<sub>3</sub>, and Mixtures of the Two, Showing Recovery of Iron Added to Blood*

Sample No.	Blood	0.1 M FeCl <sub>3</sub>	Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> used	Corrected for blank	× factor	10 <sup>-4</sup> equivalent of iron	Fe per liter of blood
	cc.	cc.	cc.	cc.			mM
1	5		4.212	4.167	1.247	5.196	10.39
2	5		4.240	4.195	1.247	5.231	10.46
3	5		4.200	4.155	1.247	5.181	10.36
4	2		1.715	1.670	1.245	2.079	10.39
5	2		1.710	1.665	1.245	2.073	10.36
6	2		1.720	1.675	1.245	2.085	10.42
7	2	3	4.125	4.080	1.247	5.088	
8	2	3	4.124	4.079	1.247	5.087	
9	2	3	4.120	4.075	1.247	5.082	
10	0	3	2.413	2.413	1.247	3.010	
11	0	3	2.413	2.413	1.247	3.010	
		Blank	0.045				
		"	0.045				
		"	0.045				
				Factor on Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub>			
		1	0.805	} 1.242			
		1	0.795				
		1	0.805				
		3	2.405	} 1.247			
		3	2.411				
		3	2.408				
		5	4.015	} 1.247			
		5	4.010				
		5	4.005				

The sum of the iron content of 2 cc. of blood, as shown in Samples 4 to 6, and the iron content of 3 cc. of the standard FeCl<sub>3</sub> solution, as shown in Samples 10 and 11 ( $2.079 + 3.010 = 5.089$ ) agrees very well with the iron content of the mixtures of these two as shown in Samples 7 to 9.

## DISCUSSION

*Necessity of Complete Oxidation of Blood*—Complete oxidation of the blood and destruction of the nitrosylsulfuric acid are necessary and are insured by the addition of H<sub>2</sub>O<sub>2</sub> and by the addition of KMnO<sub>4</sub>, as an indicator, to the aqueous solution at the end of the oxidation process. In most cases, the pink color is permanent after the first addition of KMnO<sub>4</sub>. In cases where it was not permanent and a further addition of HNO<sub>3</sub> was made, the second addition of KMnO<sub>4</sub> never failed to give a pink color which was permanent. If more than two additions of KMnO<sub>4</sub> are made, the final Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> titration value will be appreciably increased, and it will be necessary to apply a correction which is determined by the use of appropriate blanks.

If oxidation is incomplete, the colloidal suspension of Fe(OH)<sub>3</sub> is difficult to break and quantitative separation of Fe(OH)<sub>3</sub> on the filter paper is not possible. Occasionally, the alkaline filtrate from the Fe(OH)<sub>3</sub> is clear but slightly yellow, indicating, probably, incomplete oxidation, although in the few cases where such a filtrate was encountered, no test for iron was found when the filtrate was acidified and tested with potassium thiocyanate.

*Necessity of Controlling Salt Concentration and Acidity*—That sulfates, phosphates, and acidity are factors to be controlled in order to get accuracy in a thiosulfate titration is well known (4, 6), but attention has not heretofore been called to the fact that other salts, notably chlorides, exert effect. Numerous experiments on this question have led to the following conclusions.

If too much acid is present (for example 39 cc. of N HCl in 50 cc. of solution, the minimal amount necessary to dissolve and wash the ferric hydroxide from the filter paper), the starch-iodine color is purple instead of blue, and agreeing values among duplicates cannot be realized. Therefore, part of this excess acid must be neutralized by NaOH.

For a given amount of ferric ion, the amount of thiosulfate necessary to reach an end-point increases with increasing sulfate concentration and also with increasing acidity; but the thiosulfate required decreases as chlorides are increased, NaCl having a greater effect than the same weight of NH<sub>4</sub>Cl. These effects of salts and acids on the thiosulfate titer are much greater when ferric ion is being determined than when KIO<sub>3</sub>·HIO<sub>3</sub> is the oxidizing agent and iron is absent.

The amount of thiosulfate required per unit amount of iron (at controlled acidity and salt content) varies with the amount of iron, being smaller with larger amounts of iron. Thus the factor on the thiosulfate increases as the amount of iron increases. The magnitude of this effect has varied considerably in different experiments, although its direction has always been the same (see Table I). It may be that further study would allow one to control the factor on the thiosulfate and make it agree with that found with a  $\text{KIO}_3 \cdot \text{HIO}_3$  standardization; until then, however, we feel it safer to carry out our standardization with standard  $\text{FeCl}_3$  solution, keeping acidity and salt content exactly the same in both standards and unknowns, and to calibrate the factors on the thiosulfate for each blood sample according to the quantity of thiosulfate used for that sample.

*Details of Attaining End-Point with  $\text{Na}_2\text{S}_2\text{O}_3$* —It was found that when  $\text{Na}_2\text{S}_2\text{O}_3$  was added to the  $\text{FeCl}_3$  solution as soon as possible after the addition of KI, the end-point, although sharp, did not persist for more than a few seconds, as was evidenced by the reappearance of the blue color. When the solution was titrated to an end-point with starch and was then allowed to stand 5 minutes and again titrated to the end-point, the blue color did not reappear within 45 minutes to 2 hours. The second end-point was taken as the final one in these cases. It was thought that the reaction between the ferric and iodide ions was incomplete after the first addition of  $\text{Na}_2\text{S}_2\text{O}_3$  had been made and that the reappearance of color after the first or preliminary end-point was due to the completion of this reaction; so, a study was made of the effect of allowing various time intervals to elapse after the addition of the KI, before the  $\text{Na}_2\text{S}_2\text{O}_3$  was added. The results (not detailed here) showed that the reaction between the ferric and iodide ions is a slow one, and noticeably incomplete in 2 minutes, in the presence of small amounts of iodine such as are present in reaching the preliminary end-point with the iodine as an indicator. The reaction becomes complete for practical purposes by extending the time interval to 5 minutes in the presence of these minute amounts of iodine; or the iodine may be completely removed by attaining the preliminary end-point in the presence of starch, in which case completion of the reaction is practically immediate. From these studies, we decided to add starch in attaining the preliminary end-point.

Studies were also made to find out whether a larger concentration of KI would serve to decrease the time of the reaction. With 10 cc. (instead of 5 cc.) of 20 per cent KI the reaction was complete at the preliminary end-point (that is, no additional color appeared on standing), but the magnitude of the titration values increased progressively as the time interval varied from 15 seconds to 10 minutes. When, then, still larger amounts of KI were used (5 and 10 cc. of 40 per cent KI) in order to see whether these titers could be made to reach a constant maximum in a short time, a red, rather than a blue, appeared on the addition of starch, and this color was not readily discharged during the further addition of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>.

From these studies it was concluded that, for the best accuracy, 5 cc. of 20 per cent KI should be used in solutions containing the iron equivalent of 5 cc. of blood or less, that the solutions should be allowed to stand exactly 2 minutes before the Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> is added, and that after reaching a preliminary end-point in the presence of starch, 5 minutes be allowed before observing the final end-point.

*Accuracy of Method*—Table II shows that the maximum difference among triplicates is 0.1 mm when the total hemoglobin is about 10 mm. To obtain this accuracy, the maximum error of any determination should not be greater than 1 part in 200 and that this is realizable seems justified from the following considerations. The error in sampling need not be greater than 1 part in 1000, and the error in the Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> titration not greater than 0.01 cc. when the total number of cc. is between 3 and 5, or 1 part in 300 to 500. The other most likely sources of error are incomplete oxidation, loss of material during the frequent transfers and during the oxidation of blood, and inequality of salt or acid concentration in the solution during the titration. These can all be kept below 1 part in 200 by careful manipulation. While the maximum difference among triplicates in Table II is 1 part in 100, the average error is less than one-half of this, as has been borne out by other observations with this method.

#### *Correlation of Iron Content and Oxygen Capacity*

Table III shows the extent to which mm per liter of O<sub>2</sub> determined by the Van Slyke-Neill method agree with mm per liter of

iron determined by the iodometric method presented above. The values for mm of iron are averages of triplicate determinations, except in such instances as Sample AN, where one of the triplicates varies from the other two by more than 0.1 mm, in which case the iron value is the average of duplicates. The iron values are from -0.01 to +0.13 mm different from the O<sub>2</sub> values,

TABLE III  
*Comparison of O<sub>2</sub> Capacity and Iron Content of Dog Blood*

Sample	O <sub>2</sub> "	Fe	Average Fe	Difference between Fe and O <sub>2</sub>
	<i>mm</i>	<i>mm</i>	<i>mm</i>	<i>mm</i>
A, May 26	10 10	10.23 10.25 10.23	10.23	+0.13
V, " 26	9.90	9.92 9.94 9.96	9.94	+0.04
AN, " 30	9 16	9.36 9.16 9.13	9.15	-0.01
CE, " 30	8.39	8.42 8.45	8.44	+0.05
June 19	10.28	10.39 10.36 10.42	10.39	+0.11
" 19	10.28	10.46 10.39 10.36	10.40	+0.12

The O<sub>2</sub> capacity values are corrected for 0.25 mm of dissolved O<sub>2</sub> (7).

showing that within the limit of experimental error the two values agree.

#### SUMMARY

An iodometric method for the accurate determination of iron in blood is described by which the iron content may be determined to 0.1 mm of iron per liter of blood. It is shown that the following conditions must be observed in order to attain this accuracy: (1) Care must be taken to insure complete oxidation of the blood. (2) For the Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> titration, sulfates and phosphates must be removed, the acidity must not be too great, and the acidity and



salt concentration must be accurately controlled. This is accomplished by precipitating the iron as  $\text{Fe}(\text{OH})_3$ , washing until salts are completely removed, redissolving the iron in a measured excess of  $\text{HCl}$ , and partially neutralizing the acid with a measured amount of  $\text{NaOH}$ . (3) The factor on the  $\text{Na}_2\text{S}_2\text{O}_3$  must be determined by titration against standard  $\text{FeCl}_3$ , the same conditions of salt and acid concentrations as in the unknowns being used. No other standard may be used because the factor on the  $\text{Na}_2\text{S}_2\text{O}_3$  varies with the amount of iron. (4) The end-point must be determined in exactly the same way in all samples, standard and unknown, with respect to the time intervals allowed and the amounts of  $\text{KI}$  used. A preliminary end-point is reached in the presence of starch, and after standing 5 minutes, the final end-point is attained.

The iron content and O<sub>2</sub> capacity of blood were found to agree within 1 per cent.

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## THE METABOLISM OF SULFUR

### XXIII. THE INFLUENCE OF THE INGESTION OF CYSTINE, CYSTEINE, AND METHIONINE ON THE EXCRETION OF CYSTINE IN CYSTINURIA\*

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The recognition of methionine as an important building stone of the protein molecule is one of the most significant advances in our knowledge of the chemistry of the biologically important sulfur compounds (1). Methionine has been shown to function similarly to cystine both for purposes of growth in rats fed a diet inadequate in its cystine content (2) and for the detoxication of monobromobenzene (3). Thus a close relationship, in function, at least, between these two amino acids of the protein molecule may be assumed.

The recent studies of Brand and associates, which are concerned with the fate of methionine, cystine and cysteine (4), and homocystine and homocysteine (5) in the cystinuric individual, have furnished still further evidence of the interrelationship of the various sulfur-containing amino acids and have indicated a possible origin of the cystine excreted in this perplexing error of metabolism. It has been difficult to correlate the failure of cystine, when fed to a cystinuric patient, to increase the urinary cystine and its ready oxidation to and excretion as sulfate with the increased elimination of urinary cystine which has been observed repeatedly to occur when a diet high in its content of protein (and presumably cystine) has been fed (6). Brand (4)

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observed an increase in urinary cystine after oral administration of cysteine or *dl*-methionine to a cystinuric boy, while, in agreement with earlier observations, the administration of cystine did not alter the elimination of urinary cystine. On the basis of these findings, he has postulated that cysteine is a product of the catabolism of methionine and that the error in cystinuria is a failure of the proper utilization of cysteine and not of cystine. The "extra" cystine excreted after the feeding of methionine is thus considered to be derived directly from the degradation of the molecule of methionine in metabolism, cysteine being an intermediary product, a chemical transformation difficult to picture in the light of known mechanisms of catabolism of amino acids in the animal organism.

Andrews and Randall (7), on the other hand, were unable to detect any significant increase of urinary cystine after the oral administration of *dl*-methionine to a cystinuric boy and emphasize the possibility of variation between cystinuric individuals and the "desirability of experimentation with as wide a range of subjects and conditions as possible."

The observations of Brand and coworkers are important in relation to the general problem of the intermediary metabolism of the sulfur-containing amino acids and, in addition, point to the advisability of a reconsideration of dietary therapeutic measures in connection with cystinuria and the formation of cystine calculi. It has seemed desirable, therefore, to present our own metabolic studies with a cystinuric in which the level of dietary protein was varied and the influence of this factor on the excretion of "extra" cystine after the administration of the several naturally occurring sulfur-containing amino acids was determined.

#### EXPERIMENTAL

The subject of the experiments, S.P., was a 21 year-old male of Jewish parentage, 63 kilos in weight, whom we have studied at intervals since the fall of 1930, when he entered the University of Michigan (8). No history of calculus formation is obtainable, although the urine has contained "showers" of crystals almost uniformly. At no time has the subject received sodium bicarbonate or other alkaline salts. Two series of carefully controlled metabolic studies were carried out, separated by an interval of approximately 1 year, the first (1934 series) of 55 days duration

and the second (1935 series) of 50 days duration. During the experimental periods, the daily routine of the individual was very uniform, the chief exercise consisting in walking twice daily to and from the University Hospital, a total distance of approximately 5 miles, with occasional brief periods of swimming. We believe that the constancy of the daily creatinine output reflects the uniformity of the daily routine.

*Diet*—Throughout each series, we were fortunate in securing the cooperation of the Department of Dietetics of the University Hospital. The patient ate a uniform breakfast daily at a campus restaurant and received the other two meals at the Dietetic Out-patient Department of the University Hospital.<sup>1</sup> Three daily menus for each period were prepared, each containing essentially the same types of foods and supplying the same amount of protein and calories but varying slightly in the type of vegetable, etc., or meat when this was included in the diets. These three menus were used in regular rotation throughout the series, all food received being carefully weighed.

The periods of the 1934 series were three in number, characterized dietetically as follows: a moderate protein meat-free diet (56 gm. of protein, 2670 calories), a high protein diet in which the extra protein was supplied mainly by meat, cheese, and eggs (124 gm. of protein, 2720 calories), and a second moderate protein meat-free diet furnishing essentially the same amount of protein as the first diet but of slightly lower calorific value (2210 calories). In the 1935 series, the diets of the two periods were the same as the second moderate protein diet of the 1934 series and the high protein diet of the same series.

*Methods*—Creatinine was determined as a control of uniformity of routine procedures and completeness of collection of the daily urinary sample, but since the values showed no variations of significance, they are not reported in the tables. Total nitrogen was determined by the usual modified Kjeldahl method and the partition of sulfur gravimetrically, by the methods of Folin (total sulfate) and Denis (total sulfur).

For the determination of cystine in the urine, the Lugg-Sulli-

<sup>1</sup> We wish to express our appreciation of the cooperation of Miss Mabel McLachlan, director of dietetics at University Hospital, and our indebtedness to Mrs. Genevieve Cartmill O'Rourke, who supervised the dietary of our subject.

van procedure (9) with slight modifications was adopted. Since the urines always contained some cystine crystals (10), although in variable amounts, the daily samples of urine were filtered by suction through a glass funnel fitted with a sintered glass plate. The crystals were washed carefully with cold water, the washings being used to dilute the filtered urine to the desired volume, the cystine was dissolved in 3 per cent hydrochloric acid, and the determinations were carried out on this solution. Aliquots of the filtered urine were made alkaline with sodium hydroxide (pH, 12.5 (orange GG)) and the precipitated phosphates were removed by centrifugation. Aliquots of the centrifugate were acidified with hydrochloric acid (pH, approximately 1 to 2) diluted to volume and the cystine was determined by the Lugg procedure, as described for protein hydrolysates.<sup>2</sup> It was advisable to use that amount of treated urine which would contain 1.0 to 2.0 mg. of cystine. By the method outlined, excellent duplicates were obtainable. Cystine added to normal or cystinuric urines in amounts equivalent to 0.5 to 1.0 mg. per cc. of urine could be recovered with a maximum error of  $\pm 4$  per cent and an average error of less than  $\pm 2$  per cent. All the cystine determinations were made by the same analyst (B. H. B.).

Best results were obtained when the cystine determinations were made within 3 hours after the sample was received in the laboratory and diluted to volume. More stable solutions for analysis could be prepared by the norit treatment of the urine as described by Virtue and Lewis (11).

In our hands, the Lugg modification gave results which were more consistent and satisfactory than the original Sullivan procedure, although the colors obtained were less intense. Moreover, as pointed out by Brand (5) and confirmed by us, homocystine, a possible intermediate in the metabolism of methionine, while it gave no color by itself, depressed the color formation in the Sullivan method, but failed to influence the development of color in the Lugg modification.

*Compounds Administered*—Cystine from two biological sources was fed. In the earlier series (1934) the cystine was obtained from human hair; in the later series (1935) the cystine used was

<sup>2</sup> The total daily cystine excretion was calculated as the sum of the cystine of the sediment and that in solution in the urine.

prepared by purification and recrystallization of the cystine which separated spontaneously from the urine of the patient on standing (10). Both samples showed a satisfactory degree of purity on analysis. The cysteine hydrochloride was made available through the courtesy of Dr. Erwin Brand of the New York State Psychiatric Institute and Hospital. The methionine (*dl*-) was a synthetic product. The compounds to be administered were placed in capsules and half of the amount to be fed was given with the morning meal and the remainder with the midday luncheon.

TABLE I

*Average Daily Excretions of Nitrogen and Sulfur in Cystinuria during Control Periods in Which Levels of Protein Intake Varied*

Series	Protein level	Days averaged	Total N	Sulfur					Cystine
				Total	Total sulfate	Organic	Cystine	Non-cystine organic	
			gm.	gm.	gm.	gm.	gm.	gm.	gm.
1934	Moderate	6	7 7	0.66	0.39	0.27	0.18	0.09	0.66
	High	11	15 0	1.03	0.70	0.33	0.22	0.11	0.84
	Moderate	7	8 1	0.63	0.36	0.27	0.19	0.08	0.73
1935	"	12	8 2	0.64	0.35	0.29	0.20	0.09	0.77
	High	10	15 8	1.08	0.76	0.32	0.25	0.07	0.94

### Results

In Table I is presented for each series of experiments a summary of the average daily urinary excretions during the control days in which no sulfur-containing amino acids were fed. The uniformity of the sulfur excretions and partitions is striking. In the 1934 series, when the second of the two diets of moderate protein content was fed, the daily excretion of cystine averaged 0.73 gm. with total and sulfate sulfurs of 0.63 and 0.36 gm. respectively. 1 year later, when the same diet was ingested, the comparable excretions were 0.77, 0.64, and 0.35 gm. respectively. Similar close agreement in the excretions of the two series was observed in the periods of the high protein diet. The most notable feature of the data, when the periods of moderate and high dietary protein levels are compared, is the failure to obtain an increase in cystine excretion on the high protein diet in any

TABLE II

*Influence of Oral Administration of Cystine, Cysteine Hydrochloride, and Methionine on Distribution of Urinary Sulfur in Cystinuria*

The estimated daily protein content and caloric value of the diet were 56 gm. and 2670 calories respectively for Days 1 to 24 inclusive, and 124 gm. and 2210 calories for the remainder of the experimental period.

Day	Substance fed		Total N	Sulfur					Cys-tine
				Total	Total sulfate	Or-ganic	Cys-tine	Non-cys-tine or-ganic	
		gm.	gm.	gm	gm.	gm.	gm.	gm.	gm.
1*			7.9	0.64	0.35	0.29	0.20	0.09	0.76
2*			8.5	0.65	0.36	0.29	0.20	0.09	0.77
3*			8.9	0.63	0.34	0.29	0.20	0.09	0.77
4*			8.5	0.67	0.38	0.29	0.21	0.08	0.80
5	Cystine	1.56	9.7	0.97	0.67	0.30	0.19	0.11	0.72
6	"	2.10	9.1	1.06	0.76	0.30	0.19	0.11	0.73
7			8.4	0.75	0.47	0.28	0.20	0.08	0.75
8*			8.2	0.60	0.33	0.27	0.19	0.08	0.72
9*			7.6	0.60	0.32	0.28	0.20	0.08	0.77
10*			8.2	0.63	0.36	0.27	0.21	0.06	0.78
11	Cysteine HCl	2.06	8.1	0.87	0.55	0.32	0.25	0.07	0.95
12	" "	2.88	9.3	1.18	0.83	0.35	0.28	0.07	1.04
13			9.5	0.76	0.46	0.30	0.21	0.09	0.81
14*			8.5	0.65	0.36	0.29	0.21	0.08	0.78
15*			7.7	0.63	0.34	0.29	0.21	0.08	0.79
16*			8.4	0.67	0.39	0.28	0.21	0.07	0.78
17	Methionine	2.02	8.6	0.88	0.48	0.40	0.24	0.16	0.91
18	"	2.68	7.7	1.10	0.70	0.40	0.26	0.14	0.98
19			7.5	0.77	0.47	0.30	0.22	0.08	0.83
20			8.6	0.66	0.37	0.29	0.22	0.07	0.83
21			7.8	0.58	0.29	0.29	0.21	0.08	0.81
22			8.1	0.64	0.35	0.29	0.22	0.07	0.82
23*			8.1	0.63	0.34	0.29	0.20	0.09	0.77
24*			8.2	0.62	0.34	0.28	0.20	0.08	0.77
25			10.9	0.79	0.47	0.32	0.23	0.09	0.87
26*			14.5	1.13	0.80	0.33	0.25	0.08	0.95
27*			15.7	1.04	0.74	0.30	0.24	0.06	0.90
28*			14.3	0.93	0.62	0.31	0.25	0.06	0.93
29*			16.4	1.12	0.80	0.32	0.25	0.07	0.94
30*			17.0	1.10	0.79	0.31	0.24	0.07	0.92

\* These days are included in the average of normal days for the respective periods of moderate and high protein intakes, as shown in Table I (1935 series).

TABLE II—*Concluded*

Day	Substance fed		Total N	Sulfur					Cys-tine
				Total	Total sulfate	Or-ganic	Cys-tine	Non-cys-tine or-ganic	
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
31	Methionine	2 02	15.3	1 34	0.97	0.37	0.27	0.10	1.01
32	"	2 69	14.8	1 59	1.19	0.40	0.29	0.11	1.09
33			16 7	1.16	0.81	0.35	0.26	0.09	0.97
34*			16.8	1.14	0.80	0.34	0.25	0.09	0.93
35*			17.3	1.19	0.84	0.35	0.27	0.08	1.00
36*			16 0	1.09	0.77	0.32	0.26	0.06	0.96
37*			15.8	1.00	0.66	0.34	0.25	0.09	0.93
38	Cysteine HCl	2 04	15 8	1.41	1.05	0.36	0.27	0.09	1.03
39	" "	2 98	17.2	1 60	1.23	0.37	0.29	0.08	1.09
40			15 6	1.10	0.76	0.34	0.25	0.09	0.93
41			17 0	1.15	0 80	0.35	0.27	0.08	1.01
42*			14.0	1.07	0.75	0.32	0.25	0.07	0.95
43	Cystine	1 57	17.1	1.51	1.18	0.33	0.24	0.09	0.92
44	"	2 10	17.0	1.57	1 29	0 28	0 23	0.05	0.86
45			17 1	1.18	0 81	0 37	0.24	0.13	0.92

way comparable with the increases observed in the eliminations of nitrogen, total, and oxidized (sulfate) sulfur. While the average daily excretions of these elements in the 1935 series were increased by approximately 90, 70, and 115 per cent, as compared with those of the periods of moderate protein intake, the cystine excretion was increased only 22 per cent. Similar differences are to be observed in the data of the 1934 series. It is evident that, in our patient, no direct relationship between the level of excretion of total sulfur and that of cystine existed.

Several explanations of the failure of the cystine excretion to exhibit variations directly parallel to the changes in the nitrogen and total sulfur eliminations are suggested. If the urinary cystine is not related primarily to the cystine content of the diet, but is of endogenous origin to a considerable extent, as suggested by one of us (12), a direct relationship between nitrogen, total sulfur, and cystine would not be expected. If, as suggested by Brand (4, 5), the source of the cystine of the urine is some compound other than cystine, *e.g.* methionine, an increase in the level of



dietary protein would not of necessity correspond to a proportionate dietary increase in the precursor of cystine. Unfortunately, the analytical data available do not permit calculation of the relative amounts of the two sulfur-containing amino acids in our diets. It is possible that, in our high protein diet, the protein supplied may have been such that the increase of dietary cystine, which is oxidized, as we believe, by the cystinuric and leads to an increased excretion of sulfate sulfur, may have been greater than that of methionine, which in its metabolism in the cystinuric has been said to give rise to extra cystine. Under these conditions the changes of the cystine sulfur of the urine would hardly be expected to be related directly to those of total and sulfate sulfur.

It is possible also that in the cystinuric the intermediary reactions which lead to the formation of cystine may be influenced by the general level of protein metabolism, that the conversion of methionine or some other precursor to cystine is less complete when the protein catabolism is at a higher level than at a lower level. We believe that our studies in which cysteine, cystine, and methionine were fed furnish support for such a consideration.

*Cystine and Cysteine*—In Table II are presented detailed results of the 1935 series. It will be noted not only that administration of cystine (isolated from urine) failed to increase the excretion of urinary cystine, but that there appeared a slight tendency to a decreased excretion (Days 5, 6, 43, and 44). While, in view of many unsolved problems involved in the determination of cystine in urine, we do not wish at this time to emphasize these slight variations, we have observed such a tendency in all of the three experiments in which cystine, of either urinary or protein origin, was fed. On the other hand, in confirmation of most of the similar studies of other workers, the sulfate (oxidized) sulfur was materially increased, an excretion of extra sulfate sulfur equivalent to approximately 86 and 102 per cent respectively, for the two cystine periods of this series (Table III). When cysteine hydrochloride was fed (Days 11, 12, 38, and 39), an increased cystine excretion clearly greater than the experimental error of the Lugg procedure was obtained. Although there was observed also a rise in the sulfate sulfur, the increase was not as marked as with cystine, the excretion of extra sulfate sulfur corresponding to 79 and 75 per cent of the cysteine derivative fed (Table III).

TABLE III

*Extra Sulfur and Extra Cystine Excretion in Experimental Periods in Which Various Sulfur Compounds Were Fed to a Cystinuric*

Unless otherwise indicated, the sulfur-containing compounds were fed on 2 successive days, the amount given representing the total amount ingested. The excretion of extra sulfur and cystine is calculated for the 2 days in which the compounds were fed and the day immediately following. When the excretion of extra cystine was prolonged beyond this period, this is indicated by foot-notes.

Series	Dietary protein	Substance fed	Extra sulfur								Extra cystine
			Fed	Excreted as							
				Total		Sulfate		Cystine			
				gm.	per cent	gm.	per cent	gm.	per cent		
1934	Moderate	Cystine (protein)	2.50	0.38	56.7	0.30	44.7	0.00	00.0	gm.	0.00
1935	"	" (urine)	3.66	0.98	87.6	0.85	86.7	0.00	00.0	gm.	0.00
1935	High	"	3.67	0.98	1.02	1.00	102.0	0.00	00.0	gm.	0.00
1934	Moderate	Methionine	3.63	0.78	0.53	0.23	29.4	0.13	16.6	gm.	0.51*
1934	High	"	3.69	0.79	0.85	0.75	94.9	0.12	15.1	gm.	0.46
1935	Moderate	"	4.70	1.01	0.83	0.60	59.4	0.11	10.9	gm.	0.41†
1935	High	"	4.71	1.01	0.85	0.69	68.3	0.06	5.9	gm.	0.25
1934	Moderate	" †	3.00	0.64	0.53	0.31	48.4	0.09	14.0	gm.	0.33§
1934	High	" †	3.00	0.64	0.71	0.62	96.8	0.04	6.2	gm.	0.14
1935	Moderate	Cystine HCl	4.94	1.00	0.89	0.79	79.0	0.13	13.0	gm.	0.49
1935	High	"	5.02	1.01	0.87	0.76	75.2	0.06	5.9	gm.	0.23

\* Excretion of 0.37 gm. of extra cystine in 4 subsequent days, a total of 0.88 gm. of extra cystine, equivalent to 29.4 per cent of the sulfur fed.

† Excretion of 0.15 gm. of extra cystine in 3 subsequent days, a total of 0.56 gm. of extra cystine, equivalent to 14.8 per cent of the sulfur fed.

‡ The methionine was fed for 1 day only.

§ Excretion of 0.15 gm. of extra cystine in 3 subsequent days, a total of 0.48 gm. of extra cystine, equivalent to 20.3 per cent of the sulfur fed.

*Methionine*—When methionine was fed not only was there observed an increased excretion of extra cystine (Days 17, 18, 31, and 32, Table II), but as reported by Brand (4), the excretion was prolonged over several days, particularly during the period of the moderate diet (e.g., the period immediately following Days 17 and 18). The excretion of extra sulfate sulfur was not nearly as great as in the experiments with cysteine and cystine, the increases corresponding to 59 and 68 per cent respectively (Table III). Results with methionine comparable to those in Table II were also obtained in the 1934 series, not only when methionine was fed on 2 successive days, but also when slightly larger amounts of methionine were fed on a single day. The results of all the studies made are summarized in Table III. In all cases in which methionine was fed (six experimental periods), the excretion of extra cystine was distinctly greater when a diet of moderate protein content was fed than when a higher protein diet was used. Moreover, under conditions of a moderate protein diet, the excretion of extra cystine after methionine administration was prolonged over a period of several days, as noted in Table II and in the foot-notes to Table III. Thus, in the 1934 moderate protein series, extra cystine equivalent to 29 per cent of the sulfur fed was excreted over a total period of 7 days, including the experimental days, while with a high protein diet in the same series, the extra cystine corresponded to only 15 per cent of the methionine fed and the excretion of cystine returned to normal on the 2nd day after the experimental period. This value for extra cystine (29 per cent) is the greatest we have observed. All the other methionine experiments (Table III) show the same type of difference between the metabolism after methionine with high or low protein diets. Associated with this lower excretion of extra cystine sulfur on high protein diets was an increased excretion of extra sulfate sulfur when methionine was fed during the high protein period. Though the values are not entirely uniform, they all point to the same conclusion of a more satisfactory utilization (oxidation) of the sulfur of methionine on a level of high dietary protein than on the moderate protein level.

Unfortunately the amount of cysteine hydrochloride available

permitted only two series of experiments, one period with each of the two types of protein diet. The results obtained after cysteine administration also indicated a greater excretion of extra cystine on the moderate dietary protein level than on the high protein level (Tables II and III). In neither series was the excretion of extra cystine prolonged beyond the experimental period, as in the corresponding methionine experiments.

#### DISCUSSION

In general, our findings are in confirmation of those of Brand and coworkers (4), in that we also have been able to demonstrate an increased cystine excretion after feeding either methionine or cysteine to a cystinuric subject. The increases, however, are not as great as those observed by them. This may have been due to the fact that the amounts of the amino acids fed by us were somewhat less than those fed in their experiments. It should also be pointed out that we have made use of the Lugg-Sullivan procedure rather than that of Folin or Sullivan, a procedure which appears to be less susceptible to errors occasioned by the presence of  $-S-S-$  and  $-SH$  compounds other than cystine. Although it is not stated directly, the most striking results of Brand ((4) p. 76, Table IV) appear to be calculated from the values obtained by the Folin method, a method which, as shown by Brand subsequently (5), gives results somewhat too high in the presence of the products of the demethylation of methionine. Moreover, we have no basis for the assumption that the error of cystinuria is quantitatively the same in all subjects. It should be recalled that Andrews and Randall (7) observed no significant increases in cystine excretion after feeding methionine to their subject.

It has been postulated that the cystine excreted in cystinuria originates from the homocysteine formed in the demethylation of methionine or from cysteine (4, 5). It is difficult to picture such a transformation of homocysteine to cysteine and to cystine in the light of any known facts concerning the degradation of the biologically important sulfur-containing compounds either *in vitro* or *in vivo*. Until further evidence is available we are unwilling to accept such a theory of the origin of the urinary cystine,

although we are not prepared to suggest an alternative theory by which the increase in cystine excretion, which results from methionine feeding, may be explained.<sup>3</sup>

We believe, however, that, as the level of protein metabolism is increased, the ability of the cystinuric organism to utilize normally (*i.e.*, oxidize to sulfate) the sulfur-containing complex which gives rise to cystine in the urine of the cystinuric is also increased. In support of this, we recall the lower excretion of extra cystine and the higher excretion of extra sulfate sulfur after methionine feeding when our patient was receiving a high protein diet. Also as already pointed out, such a theory would explain the failure of the cystine excretion to increase in direct relation to the increase in total sulfur, when the excretions on moderate and high protein diets are compared (Table I).

We have never observed, during the experimental periods, a positive nitroprusside test in the absence of cyanide, a test which would indicate the presence of cysteine or a thiol group. We have attempted repeatedly to obtain evidence for the presence of a cystine-containing complex in the urine of our patient (7, 14). We have hydrolyzed the urine with acid and have allowed it to "age" as described by Brand and coworkers (14) and have analyzed for cystine both by the Sullivan method, as used by them, and also by the Lugg-Sullivan procedure described earlier in this paper. We have thus examined urines within 15 minutes after voiding as well as urines which have stood in the laboratory for several hours. We have never been able to detect cystine in combined form or cystine that reacted more slowly than the usual form of cystine.

In conclusion, we wish to point out again the desirability of exact specific methods by which to determine cystine satisfactorily in the presence of other possible interfering substances. When and only when it is certain that the so called cystine fraction in cystinuric urine is composed entirely of cystine and that all the cystine in the presence of interfering substances is determined can we expect to approach the solution of the problem of cystinuria.

<sup>3</sup> If cysteine is present in significant quantities in many proteins as maintained by Mirsky and Anson (13), the cysteine component of the protein molecule may be in part responsible for the cystine excreted by the cystinuric under normal dietary conditions.

## SUMMARY

1. When cystine, either isolated from the patient's own urine or obtained from hydrolysis of human hair, was fed to a cystinuric patient, no extra cystine was excreted in the urine, but large amounts of extra sulfur as sulfate were excreted.

2. In similar experiments in which cysteine hydrochloride was fed, there was observed an increase in the cystine content of the urine which is believed to be far greater than the error of the analytical method used for the determination of cystine (modification of the Lugg-Sullivan procedure). A considerable excretion of extra sulfate sulfur was also obtained.

3. When *dl*-methionine was fed, extra cystine was also excreted and the increase of sulfate sulfur was less striking.

4. When the patient received a high protein diet (approximately 124 gm. of protein) the extra cystine excretion after the administration of methionine was less than that observed under similar conditions when a diet of moderate protein content (55 to 60 gm. of protein) was fed. In the latter case, the excretion of extra cystine after methionine feeding was prolonged for several days after the experimental days, while with a diet of high protein content, the cystine excretion returned to normal promptly after the cessation of the administration of methionine.

5. It is suggested that the utilization of the precursor of the urinary cystine in cystinuria occurs more readily under conditions of a high level of protein metabolism. Such a theory would explain the failure of the cystine sulfur to maintain a constant ratio to the nitrogen or total sulfur of the urine, as the amount of protein catabolized is increased.

6. In our patient, we were unable to obtain evidence of the presence in the urine of a readily split complex containing cystine.

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## **FAT METABOLISM IN PLANTS, WITH SPECIAL REFERENCE TO STEROLS**

### **II. DIFFERENTIAL CHANGES IN THE COTYLEDONS AND IN THE ROOTS, STEMS, AND LEAVES**

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In a recent paper the author (1) has shown that during the germination of the soy bean, while the total fat content diminished markedly, there was a continuous synthesis of sterol which was usually somewhat greater during etiolated growth than during growth in the light. These results were in fair agreement with those reported by Beumer (2) for germinating peas and beans. It was also observed that during the period of rapid fat mobilization and utilization there was a marked esterification of the sterol, which suggests that there is a close relation between the sterol metabolism and the fat metabolism in plants. These changes have, however, been observed in the ether extract of the entire seedling. In order to understand the significance of the changes in the oil reserve in the metabolism of the seedling, the changes in the oil of the cotyledons, *i.e.* the reserve stores, must be considered separately from the oily material of the roots, stems, and leaves. With this in mind experiments were carried out to determine the changes in the fatty acid and sterol contents of the soy bean during germination in the light and in the dark, the cotyledons being removed from the young plants and the two parts analyzed separately.

#### **EXPERIMENTAL**

Sound soy beans (black Wilson) were germinated in the light and in the dark, at different times of the year (June and November) in a manner similar to that described in an earlier paper (1).



At the end of 2 weeks the seedlings were removed from the germinators, the cotyledons carefully separated from the young plants, and the separate parts analyzed according to the methods previously described: total fatty acids by the oxidative methods of Bloor (3); iodine numbers of the fatty acids by the method of Yasuda (4); total and free sterol by the method of Okey (5) as modified by Yasuda (6) and by others (see Boyd (7)). From the values obtained by the above experimental procedures the further distribution of the lipids was found by calculation.

### *Results*

In Table I are presented the results of the differential analysis of (a) the roots, stems, and leaves, and (b) the cotyledons of soy beans germinated in water for 2 weeks in the light and in the dark. (The values for ungerminated seeds are included for comparison.) The values are expressed on the basis of dry weight. In addition (Table II) the percentage changes in the sterol content are calculated in terms of the amount originally present in the ungerminated seeds, since the dry weight of the seeds showed a decrease of  $12.3 \pm 1$  and  $13.6 \pm 2$  per cent during germination in the light and in the dark, respectively.

The results of the lipid analysis of seeds germinated at different seasons of the year (June and November) are very similar. The same is true of seeds whether germinated in the light or in the dark, except for the total fatty acid content of the cotyledons. The moisture content shows an increase from 6.74 per cent in the ungerminated seeds to 93 to 95 per cent in the roots, stems, and leaves, but only 78 to 83 per cent in the cotyledons. The total fatty acids, about 17 per cent in the ungerminated seeds, show a decrease to 6.5 per cent in the cotyledons in the light and 10.3 per cent in the dark. On the other hand, whether the plants were grown in the light or in the dark, the fatty acid content of the roots, stems, and leaves is quite similar, amounting to about 2.5 per cent of the dry weight. The iodine numbers of the fatty acids of the cotyledons in both the light and the dark are of the same order as those of the ungerminated seeds, namely about 133, while those of the roots, stems, and leaves are about 30 points lower.

Calculated on the basis of dry weight, the total sterol (Table I) shows an increase from 97 mg. per cent before germination to

TABLE I

*Results of Differential Analysis of Roots, Stems, and Leaves, and Cotyledons of Soy Beans Germinated in Water for 2 Weeks in the Light and in the Dark (Calculated on Basis of Dry Weight)*

The values for ungerminated seeds have been included for comparison.

Sample No.	Time of germination	No. of plants	Moisture	Total fatty acids		Sterol	
					Iodine No.	Total	Free in total
Roots, stems, and leaves							
Ungerminated seeds (average of 6 samples)			per cent 6.74±0.30	per cent 16.8 ±0.3	133±4	mg. per cent 97±2	per cent 84±3
Seeds germinated in light							
41	June 5-18	292	94	2.71	118	189	86
44	Oct. 28-Nov. 9	190	93	2.63	97	177	92
45	" 28- " 9	210	93	2.64	97	184	98
46	" 28- " 9	160	93	2.77	104	206	83
		852	93±0	2.69±0.05	104±7	189±9	90±5
Seeds germinated in dark							
43	June 5-18	310	95	2.43	110	192	82
47	Oct. 28-Nov. 9	200	95	2.47	98	183	89
48	" 28- " 9	210	95	2.44	101	208	91
49	" 28- " 9	200	95	2.52	98	230	82
		920	95±0	2.47±0.03	102±4	203±21	86±4
Cotyledons							
Seeds germinated in light							
41	June 5-18	292	83	5.45	144	221	35
44	Oct. 28-Nov. 9	190	83	7.18	133	212	41
45	" 28- " 9	210	83	5.94	133	210	39
46	" 28- " 9	160	82	7.37	132	215	41
		852	83±0	6.49±0.79	135±4	215±4	39±2
Seeds germinated in dark							
43	June 5-18	310	73	11.35	137	191	43
47	Oct. 28-Nov. 9	200	78	10.90	131	212	42
48	" 28- " 9	210	80	9.73	132	243	39
49	" 28- " 9	200	79	9.30	133	225	40
		920	78±2	10.32±0.80	133±2	218±16	41±2

about 200 mg. per cent in both the cotyledons and in the roots, stems, and leaves of seedlings grown in the light and in the dark. However, as pointed out above, the dry weight of the seedlings decreased considerably during germination, which causes an undue increase in the values for the total sterol when based on the dry weight. In order to get a true conception of the increase in the sterol content during germination, the values should be calculated on the basis of the amount originally present in the ungerminated seeds. The figures are given in Table II. In agreement with earlier findings the total sterol of the whole plant shows an actual

TABLE II

*Partition of Percentage Increase in Sterol Content of Bean Seedlings, Based on Amount of Sterol Originally Present in Ungerminated Seeds*

	Sample No	Whole plant (W)	Cotyledons (C)	Roots, stems, and leaves (W - C)
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Seeds germinated in light	41	88	10.9	77.1
	44	80	2.4	77.6
	45	81	4.7	76.3
	46	88	8.2	79.8
Average .....		84±4	6.5±3	77.7±1
Seeds germinated in dark	43	76	9.5	66.5
	47	82	11.8	70.2
	48	101	14.3	86.7
	49	96	8.4	87.6
Average .....		89±10	11.0±2	77.8±9

increase of 85 to 90 per cent, being slightly though perhaps not significantly greater during germination in the dark than in the light. It is also evident from Table II that by far the greater part of this increase occurred in the roots, stems, and leaves, the amount in the cotyledons being only slightly greater after germination than before.

Concerning the relation of the free to the ester sterol, a marked difference is evident in the different parts of the plants grown both in the light and in the dark (Table I). In the roots, stems, and leaves the relation is not unlike that in the ungerminated seeds,

about 85 to 90 per cent of the sterol being present in the free form. In the cotyledons, on the other hand, the relation is quite different, only about 40 per cent of the sterol being present in the free form.

#### DISCUSSION

The lipid analysis of seeds germinated under constant environmental conditions shows no dependence on the season of germination (June or November).

In discussing the changes in the fat content of germinating seedlings one should distinguish between the temporary fat reserves in certain tissues, *e.g.* cotyledons, and the frequent, perhaps invariable, occurrence of smaller quantities of fatty substances in other tissues, *e.g.* roots, stems, and leaves. Evidence from the literature indicates that oil is rarely if ever moved from one region of a plant to another (Miller (8)). The fatty acid values for the cotyledons (Table I) therefore represent the portion of the original fat reserve of the seed still unused. These values, in agreement with earlier findings (1), indicate that there has been a greater utilization of fat during germination in the light than in the dark. Although the reason for this is not clear, it has been shown by Pierce, Sheldon, and Murlin (9) and others that during the germination of oleaginous seeds the fat stored in the seed is converted to carbohydrate. Since the decrease in the dry weight of the entire seedling germinated in the light and in the dark is of the same order, the greater decrease in the amount of fat in the former is possibly due to a greater amount of conversion of fat to carbohydrate. This suggests the interesting speculation that there may be some relation between the rate of conversion of fat to carbohydrate and photosynthetic activity in the plant. The fatty acid values for the roots, stems, and leaves, on the other hand, probably represent newly formed fatty acids. It is of interest that they are comparable in amount in the seedlings germinated in the light and in the dark.

The iodine numbers of the fatty acids of the cotyledons are similar to the values obtained for the ungerminated seeds. The fatty acids of the roots, stems, and leaves, however, are considerably more saturated. These findings indicate that there is no preferential utilization of fatty acids in the cotyledons, and that the

nature and amount of fatty acids synthesized in the roots, stems, and leaves during germination are quite independent of the rate of utilization of the reserve stores of fat in the cotyledons.

The marked synthesis and esterification of sterol reported earlier (1) for germinating soy beans during the period of rapid fat utilization are confirmed. The analyses (Tables I and II) show, however, that the synthesis of new sterol takes place chiefly in the roots, stems, and leaves of the young plant, the amount in the cotyledons being of the same order as that in the ungerminated seeds. The esterification of the sterol, on the other hand, takes place principally in the cotyledons, the newly formed sterol in the roots, stems, and leaves being present mostly in the free form. These results appear to indicate that the sterol originally present in the seed (*a*) is not used up for the development of the new plant, and (*b*) is in some way closely connected with the utilization of the fat in the cotyledons. The marked increase in the sterol content of the roots, stems, and leaves of the young plant cannot be accounted for on the basis that the sterol is a waste product, since if this were the case, a greater accumulation of sterol should be found in the cotyledons where catabolic processes are probably much greater.

The evidence would seem to justify the conclusion that in the cotyledons the sterols are involved in the metabolism of the temporary fat reserves, but in the roots, stems, and leaves the sterols represent vital tissue constituents.

#### SUMMARY

The changes in the total fatty acids and in the free and esterified sterol of soy beans were determined after germination for 2 weeks in the light and in the dark. It was found that:

1. The total fat of the cotyledons diminished markedly as germination proceeded, the decrease being more marked during germination in the light than in the dark.
2. The roots, stems, and leaves synthesized fat equally well in the light and dark.
3. Whereas the fatty acids of the cotyledons were not different in their degree of saturation from those of the ungerminated seeds, the newly formed fatty acids of the roots, stems, and leaves were considerably more saturated.

4. The marked increase in the sterol content of germinating soy beans occurred chiefly in the roots, stems, and leaves of the young plants. The esterification of the sterol, on the other hand, occurred chiefly in the cotyledons.

5. The results suggest that (a) a close relation exists between the metabolism of the sterol and the utilization of fat in the cotyledons, and (b) the sterol is a vital constituent of the roots, stems, and leaves of the young plant and not a waste product.

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# ON THE NATURE OF THE REACTION BETWEEN DIAZOTIZED SULFANILIC ACID AND PROTEINS

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It has been generally assumed since the work of Pauly (6) and of Inouye (5) that the reactivity of proteins with diazo compounds is due to the imidazole group of histidine and the phenyl group of tyrosine. There are, however, several indications that these are not the only groups concerned in the reaction. Thus, Hooker and Boyd (4) found that casein coupled with diazoarsanilic acid contained approximately 78 atoms of As in each molecule of protein. Similarly, the experiment cited below shows that each molecule of serum protein can apparently inactivate on the order of several hundred molecules of diazotized sulfanilic acid. If we take 100,000 as an "average" molecular weight for the serum proteins, and take 6 and 3 per cent for the approximate tyrosine and histidine contents, respectively (Cohn (3) p. 870); it follows that each molecule of serum protein contains on the order of twenty histidine and thirty-five tyrosine groups. Even the assumption that every imidazole and phenol group incorporated in the protein molecule remains free and reactive would not account for the observed reactivity of the protein molecule with diazo compounds.

*Preparation of Diazotized Sulfanilic Acid*—For 200 cc. of a  $m/7$  solution, 5 gm. of sulfanilic acid were treated with 1.75 gm. of anhydrous  $Na_2CO_3$  dissolved in 75 cc. of water and with 2.0 gm. of  $NaNO_2$  dissolved in 10 cc. of water. The solution was cooled in ice water and diazotized at 3–5°, a mechanical stirrer being used, by the slow addition of 35 cc. of  $N HCl$  from a separatory funnel, the tip of which was immersed in the liquid. The pale yellow, somewhat acid diazonium solution was brought to pH 7.2 to 7.6 with dilute  $NaOH$ . Since the solutions coupled



with the pH indicators ordinarily used, it was found necessary to prepare a somewhat yellowish solution of brom-thymol blue and note the evanescent color formed on adding a few drops of this to a little of the diazo solution contained in a small tube. 8 cc. of 0.5 M phosphate buffer containing 4 parts of  $\text{Na}_2\text{HPO}_4$  were then added, and the whole diluted to 200 cc.

Serially decreasing quantities of horse or human serum in a total volume of 1.6 cc. were mixed with 0.2 cc. of the neutral M/7 diazo solution. Portions of each tube were then withdrawn at intervals of 4, 16, 64, and 180 minutes and tested for free diazo compound by the addition of  $\alpha$ -naphthol in alkaline solution. The original M/7 solution of diazosulfanilate gave a vivid red color even in

TABLE I  
*Inactivation of Diazotized Sulfanilic Acid by Horse Serum*

The readings represent the color given by aliquot samples with  $\alpha$ -naphthol: + = intense red;  $\pm$  = trace of red; 0 = no color, *i.e.* no free diazo compound.

Horse serum, cc. ....	1.6	0.8	0.4	0.2	0.1	0.05	0.025	0.0125
$\text{H}_2\text{O}$ , cc. ....		0.8	1.2	1.4	1.5	1.55	1.6	1.6
M/7 sodium diazosulfanilate, cc. ....	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
4 min. ....	0	0	$\pm$	+	+	+	+	+
16 " ....	0	0	0	$\pm$	+	+	+	+
64 " ....	0	0	0	0	+	+	+	+
3 hrs. ....	0	0	0	0	0	$\pm$	+	+

a 1:8000 dilution on the addition of the  $\alpha$ -naphthol. It follows that in any experimental tube which failed to develop color, more than 99.9 per cent of the diazo compound had been inactivated by the protein.

As is shown in Table I, approximately 0.10 cc. of horse serum completely inactivated 0.2 cc. of M/7 diazo solution under the conditions of the test within 3 hours. An essentially similar result was obtained with human serum. If we take 7 per cent as the serum protein content, and assume an "average" molecular weight of 100,000 for the serum protein, it follows that  $(0.07 \times 0.10)/100,000 = 0.7 \times 10^{-7}$  moles of protein inactivated  $(0.2 \times \frac{1}{8000})/1000 = 3 \times 10^{-6}$  moles of sulfanilic acid, a ratio of 1:400.

It is true that a large and indeterminate error is introduced into this experiment, first, by the possibility that the sulfanilic acid

TABLE II  
*Reactivity of Various Groups Present in Protein with Sodium Diazosulfanilate*

The readings represent the color given by aliquot samples with  $\alpha$ -naphthol: + = intense red;  $\pm$  = trace of red; 0 = no color, i.e. no free diazo compound. Varying quantities of the various substances in m/7 solution were mixed with 0.1 cc. of neutralized m/7 diazosulfanilic acid. The mixtures were tested for free diazo compound after  $\frac{1}{2}$  hour at room temperature by the addition of  $\alpha$ -naphthol in alkaline solution.

Group	Substance taken as prototype	Cc. of substance added								Conclusion
		0.8	0.4	0.2	0.1	0.05	0.025	0.0125	0.0002	
Aliphatic NH <sub>2</sub>	Glycine	0	0	0	0	0	+	+	+	All react with diazo compound
	Alanine	0	0	0	0	0	+	+	+	
	Phenylalanine	0	0	0	0	0	+	+	+	
	Ornithine	0	0	0	0	0	0	$\pm$	+	
	Lysine	0	0	0	0	0	0	$\pm$	+	
NH of proline and hydroxyproline NH of tryptophane	Proline	0	0	0	0	+	+	+	+	Both react with diazo compound
	Hydroxyproline	0	0	0	0	$\pm$	+	+	+	
	Indole*	0	0	0	0	0	+	+	+	Indole reacts with diazo compound
NH of arginine NHCO CONH <sub>2</sub>	Guanidin†	+	+	+	+	+	+	+	+	No reactivity with diazo compound even with 48-fold excess of substance being tested
	Diketopiperazin†	+	+	+	+	+	+	+	+	
	Acetamid†	+	+	+	+	+	+	+	+	

\* Both the indole and the diazo compound were used in m/42 solution.

† Diazo compound in m/42 solution; substance in m/7 solution.

was not completely diazotized, and second, by the fact that the diazo compound is unstable in alkaline reaction. Nevertheless, the experiment strongly suggests that histidine and tyrosine are not alone responsible for the inactivation of diazosulfanilic acid by serum protein.

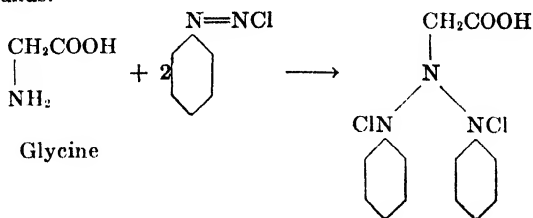
An attempt was therefore made to determine whether any groups in protein other than these two participate in this reaction.<sup>1</sup> There were chosen for study (1) the aliphatic  $\text{NH}_2$  (glycine, alanine, phenylalanine, ornithine, lysine), (2)  $\text{CONH}_2$  (acetamide), (3)  $\text{NHCO}$ , the peptide linkage (diketopiperazine), (4) indole (tryptophane), (5) guanidine  $\text{NH}$  (arginine), (6) the  $\text{NH}$  of proline and hydroxyproline.

The various substances containing some one group were dissolved and tested for reactivity with diazosulfanilate by the same technique described above. The substances which were used as prototypes for the various groups occurring in protein, and a single experiment, are summarized in Table II.

The results can be briefly summarized. As was suspected, several groups in protein other than the histidine and the tyrosine were found capable of reacting with diazosulfanilic acid. Numerically by far the most important, exceeding in fact the two groups just cited, is the aliphatic  $\text{NH}_2$  group. Glycine, alanine, lysine, ornithine, and phenylalanine all neutralize the diazo compound.<sup>2</sup> Similarly, proline and hydroxyproline inactivate the

<sup>1</sup> The finding of Boyd and Hooker (1) that a large series of amino acids failed to give demonstrable color with diazotized arsanilic acid does not exclude such participation. Substances such as diazoamino compounds, might be formed which are not significantly more colored than the diazo compound itself.

<sup>2</sup> Soon after this paper was completed, our attention was called to the fact that Busch, Patrascanu, and Weber (2) had actually isolated the compounds formed by glycine and sarcosine with benzene diazonium chloride and *p*-nitrobenzene diazonium chloride, respectively, and had identified them on the basis of their N content as the corresponding addition compounds.



diazo compound, presumably by virtue of their NH group. Finally, indole as such reacted with the diazosulfanilate, as evidenced both by the development of color and by the disappearance of reactivity with  $\alpha$ -naphthol. Tryptophane, therefore, probably reacts with diazo compounds at both the aliphatic  $\text{NH}_2$  and the indole groups.

On the other hand, neither the peptide linkage of diketopiperazine, the  $\text{CONH}_2$  group of acetamide, nor the NH of arginine (guanidine) caused any demonstrable inactivation of the diazo compound when similarly tested.

Because of the instability of the diazo compound, and the possibility of incomplete diazotization, the quantitative relationships of Table II cannot be taken to represent the actual combining proportions between the diazo substance and the various compounds with which it reacts. It is, however, to be noted that each  $\text{NH}_2$  group inactivated approximately 2 molecules of the diazo compound.<sup>2</sup>

If one is justified in assuming that the reactivity of these groups with diazo compounds is not abolished by their incorporation into the protein molecule, it follows that the reaction between proteins and diazo compounds may not be due simply to the reactivity of the histidine-imidazole ring and the phenyl group of tyrosine, but may be a far more complex reaction which involves in addition all the aliphatic  $\text{NH}_2$  groups, the indole group of tryptophane, and the NH group of proline and hydroxyproline.

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They also isolated, but were unable to identify, the compound formed by alanine with *p*-nitrobenzene diazonium chloride. It is to be noted that in the crude experiment summarized in Table II, the approximate combining ratio of 2 between the diazo compound and the several aliphatic amino acids agrees with the composition of the addition compound postulated by Busch and his coworkers on the basis of their N analyses.



## THE ACTION OF VARIOUS REAGENTS ON INSULIN\*

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In the course of our studies on the chemical nature of insulin, it became of interest to make a quantitative investigation of the behavior of the cystine and amino nitrogen of the hormone under conditions leading either to reversible or irreversible inactivation. This was necessitated by numerous qualitative observations which suggested a relationship between pharmacodynamic function and the presence of the dithio ( $-S-S-$ ) linkages and certain free amino groups in the insulin molecule (1-5). For the sake of brevity we present only a synopsis of our observations and a tabulation of the results.

### EXPERIMENTAL

While the preliminary experiments were performed with an amorphous insulin preparation (22 units per mg.), all the data here reported were obtained with crystalline insulin. Physiological standardization of the various reaction products was carried out as previously described (1). Alterations in the cystine content were followed by applying the Sullivan method (6) to acid hydrolysates of the different insulin preparations. According to Sullivan, the specificity of the method is such that reactions involving a destruction or alteration of the amino groups of the cystine molecule as well as those directly involving the dithio linkages should give lower cystine values. Our data, therefore, must be interpreted from this point of view. The amino nitrogen

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determinations were performed in a Van Slyke microapparatus. 50 mg. samples were dissolved in 5 cc. of either 0.01 N HCl or

TABLE I

*Behavior of Cystine and Amino Nitrogen of Insulin under the Action of Various Reagents*

Experiment No.	Crystalline insulin treated with	Activity*	Amino N	Cystine
		per cent	per cent	per cent
1	Untreated	100	0 97	8.5
2	N/30 NaOH, 0°, 3 hrs.	95	0 92	8.3
3	N/30 " 34°, 1½ hrs.	40		5.9†
4	N/30 " 34°, 3 hrs.	0	0 72	4.3†
5	Benzaldehyde	20	0 50	6.3
6	o-Chlorobenzaldehyde†	20	0 34	5.8
7	Formaldehyde	20	0 68	6.2
8	Isoamyl nitrite, alcohol, 3 hrs.	95	0 70	
9	Isoamyl nitrite, acetic acid, ½ hr.	95	0 50	
10	Isoamyl nitrite, acetic acid, 3 hrs.	20	0 35	
11	HCl salt, isoamyl nitrite, acetic acid, ½ hr.	20	0 25	
12	Acetic anhydride	40, § 10	0.42, § 0.10	8.6, § 7.2
13	Acid alcohol	10	1.10	8.9
14	Diazomethane	10	0 42	7.2
15	Methyl iodide	10	0 92	7.2
16	Hydriodic acid	0	0 90, 0 50	8.9, 6.0
17	Iodine	0	0 90, 0 50	6.0

\* 100 per cent activity corresponds to 24 international units.

† These data agree with those obtained by Dr. Sullivan under similar conditions (private communication).

‡ Cl content was found to be 0.24 and 0.21 per cent; crystalline insulin used gave a completely negative result (analysis carried out by Dr. Ing. A. Schoeller, Berlin-Schmargendorf, Germany).

§ Prepared according to Jensen and DeLawder (15).

|| Prepared according to Charles and Scott (18), and according to Freudenberg and coworker (12).

m/15 Na<sub>2</sub>HPO<sub>4</sub>, 2 cc. being used for an individual determination. The nitrous acid-protein mixtures were shaken for 15 minutes,

caprylic alcohol being added to prevent foaming. Since the action of nitrous acid on cystine (7) and on glutamine (8), both of which are present in insulin, may lead to higher amino nitrogen values, these figures must be interpreted on a comparative basis. The data are not without significance, however, since insulin derivatives in which the free amino groups have been blocked show a definite decrease in amino nitrogen, although the cystine content and amide nitrogen are unchanged (2). The various insulin derivatives were prepared according to the directions given in the literature to which reference is made in the discussion. Other experimental details can be found in Table I or in the discussion of the various experiments.

#### DISCUSSION

*Action of Alkali (Experiments 2 to 4)*—Insulin, on treatment with  $N/30$  sodium hydroxide for 3 hours at  $34^\circ$ , is irreversibly<sup>1</sup> inactivated with the simultaneous liberation of ammonia, which is most probably derived from certain of the free amino groups (9–13). This assumption is supported by the observation that the amino nitrogen shows a small but definite decrease after alkaline inactivation. The dithio linkages are unquestionably affected, since the decrease in cystine (to approximately half its original value) is far more than is to be expected even if all the ammonia liberated is derived from the cystine. Alkaline inactivation is not accompanied by a demonstrable change in either the tyrosine<sup>2</sup> or arginine content (11) of the hormone.

*Action of Aldehydes (Experiments 5 to 7)*—Treatment of insulin with aldehydes in faintly alkaline solution yields a compound retaining approximately 20 per cent of the original activity. While Jensen and DeLawder (15) originally ascribed this inactivation to a condensation of the aldehydes with the free amino groups of the insulin molecule, Freudenberg and his coworkers (10, 12) have suggested that inactivation can also be caused by the peroxides present in the benzaldehyde and *o*-chlorobenzaldehyde. This

<sup>1</sup> See, however, Freudenberg and Wegmann (3).

<sup>2</sup> The tyrosine content of insulin, inactivated by treatment with  $N/30$  or  $0.1 N$  sodium hydroxide for 3 hours at  $34^\circ$  has been found to be 12 per cent, a value which agrees very well with that previously reported for crystalline insulin (14).



latter view finds support in our observation that a marked decrease in cystine content occurs. The decrease of amino nitrogen, and the presence of chlorine in the reaction product from treatment with *o*-chlorobenzaldehyde, clearly indicates, however, that condensation has occurred between the aldehyde and free amino groups and also possibly other groupings in the protein molecule.

*Action of Nitrous Acid (Experiments 8 to 11)*—Freudenberg and his coworkers (10) have reported that insulin on treatment with isoamyl nitrite, either in methyl alcohol or acetic acid solution, yields an inactive product retaining approximately its original content of amino nitrogen. In repeating these experiments we have been unable to corroborate their results. We have found that insulin treated with isoamyl nitrite either in methyl alcohol (3 hours) or acetic acid ( $\frac{1}{2}$  hour) shows a definite decrease in amino nitrogen with only a slight loss in physiological activity. While precise measurement of the cystine content of these preparations is prevented by the highly colored nature of the reaction products, there is apparently no striking change in this respect. The loss of amino nitrogen under these conditions most probably results from the initial reaction of nitrous acid with those amino groups that are not concerned in the physiological action of the hormone (either the  $\epsilon$ -amino group of lysine or the free amino group of phenylalanine (2)). Prolonged action (3 hours) of isoamyl nitrite on insulin in acetic acid solution results in pronounced loss of activity with a larger decrease in amino nitrogen. It is probable that inactivation of insulin by nitrous acid takes place only after certain of the free amino groups have been displaced.<sup>3</sup> In contrast to Experiment 9, treatment of insulin hydrochloride with isoamyl nitrite in acetic acid for half an hour yields a product retaining only 20 per cent of its original activity and less than half of its original amino nitrogen value.

*Action of Acetic Anhydride (Experiment 12)*—Treatment of insulin with acetic anhydride results in an acetylated and partially inactive product, which is capable of partial reactivation by dilute alkali (12, 15, 18). We have observed that, depending on the conditions of acetylation, the cystine content of the acetylated

<sup>3</sup> It is of interest to note that similar results have been obtained in the study of the action of nitrous acid on the parathyroid hormone (16) and on secretin (17).

preparation may or may not show a decrease. If inactivation is due solely to acetylation of amino groups, the cystine value should remain unchanged unless the cystine in the acetylated insulin has become more labile towards acid,<sup>4</sup> in which case decomposition of cystine would occur during the acid hydrolysis preliminary to the cystine determination. Decrease of amino nitrogen, however, always occurs, although here again the extent of the change varies with the conditions of acetylation. It is probable that inactivation by acetic anhydride is due primarily to the acetylation of free amino groups, and is comparable to the reaction of insulin with phenyl isocyanate (2, 5).

*Action of Acid Alcohol (Experiment 13)*—We have found that insulin inactivated by acid alcohol, according to Carr *et al.* (20), shows no change in cystine content. There is some indication of a slight increase in amino nitrogen, an observation which would agree with the finding of Harvey, Howitt, and Prideaux on the acid-combining capacity of acid alcohol-inactivated insulin (21). Our original view that condensation of amino and carboxyl groups (*i.e.* anhydride formation) occurs under the influence of acid alcohol (22) must be abandoned. As Charles and Scott have suggested, the inactivation under these conditions probably involves a reversible intramolecular rearrangement (23); they were able to show that inactivation was not due to esterification.

*Action of Diazomethane (Experiment 14)*—Freudenberg and associates (10) have reported that the partially inactivated product resulting under certain conditions from the action of diazomethane on insulin is capable of partial reactivation. We have found that the insulin preparation thus obtained shows a decrease in both cystine and amino nitrogen content. The reaction of the diazomethane with dithio linkages under these conditions is suggested by preliminary observations we have made on the behavior of cystine methyl ester (used as the hydrochloride) when treated with diazomethane, although the exact nature of the process is not, as yet, clear.

*Action of Methyl Iodide (Experiment 15)*—Insulin, inactivated by the action of methyl iodide in acid alcohol solution, according to the procedure of Charles and Scott (24), yields a preparation

<sup>4</sup> It has been found that the phenylhydantoin of cystine is decomposed on heating with hydrochloric acid (19).

showing a loss in cystine content, while the amino nitrogen is unchanged. Inactivation by methylation of free amino groups therefore seems improbable. Loss of activity under these conditions may possibly occur either by oxidative destruction of the disulfide linkages by iodine formed by oxidation of methyl iodide during the reaction (see Experiment 17), or by the presence of hydriodic acid resulting from the action of methyl iodide on hydroxyl or carboxyl groups in the insulin molecule. We have found that treatment of insulin at the same temperature and for the same length of time in an alcoholic solution of 0.5 per cent hydriodic acid in a nitrogen atmosphere yields an inactive product showing no change in amino nitrogen or cystine content. In the presence of oxygen, however, a decrease in amino nitrogen and cystine content is always observed (Experiment 16). Inactivation by hydriodic acid (in a nitrogen atmosphere) is probably similar in its mechanism to that brought about by acid alcohol (Experiment 13), while inactivation in the presence of oxygen is probably due to the presence of iodine formed by the oxidation of hydriodic acid (Experiment 17).

*Action of Iodine (Experiment 17)*—Iodine in faintly alkaline solution inactivates insulin (25) with a simultaneous loss in cystine. Lowering of amino nitrogen under these conditions was also observed in several instances. While these observations favor the view that inactivation is due to a disruption of the dithio linkage and is oxidative in its mechanism, the only occasionally observed loss in amino nitrogen cannot yet be explained.

*Miscellaneous Experiments*—Many of the reagents employed in the preparation of various insulin derivatives are capable of reacting not only with the free amino groups but also with the phenolic hydroxyl groups (tyrosine) in the insulin molecule. In order to determine to what extent the phenolic groups in insulin have been affected under the influence of certain reagents (acetic anhydride, methyl iodide, and phenyl isocyanate (2)), we have compared the tyrosine content of these unhydrolyzed insulin derivatives with that of crystalline insulin, using the method of Folin and Marenzi. In general there is a slight but definite decrease in tyrosine (phenolic groups), usually not more than 20 per cent of the original value. Further work is necessary to decide whether the phenolic groups of the insulin molecule are concerned with the pharmacodynamic function of the hormone.

It has already been pointed out that insulin under the influence of weak alkali ( $N/30$  NaOH, 3 hours at  $34^{\circ}$ ) will liberate 0.1 to 0.18 per cent ammonia. We have found that under the same conditions the insulin preparations in which all or certain of the amino groups are unaffected (namely, the active isoamyl nitrite preparations, Experiments 8 and 9, and the inactive acid alcohol preparation, Experiment 13) liberate approximately the same amount of ammonia as crystalline insulin. Other insulin derivatives liberate either half this amount of ammonia or less. Attention should be called, however, to the fact that those insulin derivatives in which the cystine content is decreased, although the amino nitrogen is unchanged (Experiments 15 to 17), invariably show a decrease in the amount of ammonia liberated by alkaline treatment. The amount of ammonia liberated under these conditions is apparently dependent not only upon the presence of certain free amino groups but also upon the existence of certain dithio linkages in the molecule as well.

The formation of a heat precipitate when insulin is heated with  $0.1\ N$  HCl in a boiling water bath was first observed by du Vigneaud and coworkers (26). In applying this reaction to those insulin derivatives that are soluble in  $0.1\ N$  hydrochloric acid we have observed that the preparations, in which either certain of the free amino groups have been blocked or removed, or the dithio linkages have been disrupted, exhibit a pronounced decrease in the rate of formation of the heat precipitate, or fail to form a precipitate at all. On the other hand, we have found that those insulin derivatives in which certain of the free amino groups are apparently unaffected and in which, furthermore, the disulfide linkages are still intact (Experiments 8, 9, and 13) form a heat precipitate as readily as crystalline insulin.

Whether the discrepancy between the cystine content (12 per cent) based on the total sulfur content (3.2 per cent) and the value obtained by colorimetric determinations (Sullivan 8 to 9 per cent, Folin-Marenzi 9 to 10 per cent)<sup>6</sup> is due to the presence of an unknown sulfur constituent in insulin or to the partial destruction of cystine during acid hydrolysis of the hormone is still problemati-

<sup>6</sup> The Folin-Looney method gave values of 12 to 14 per cent as previously reported (14). Dr. Sullivan has informed us that with the Folin-Marenzi method he, also, has obtained values for the cystine content similar to those above (private communication).

cal.<sup>6</sup> The observation of Freudenberg and coworkers (9) that the test for thiolmethyl groups is negative with crystalline insulin has been confirmed by us with both hydrolyzed and unhydrolyzed protein. These observations seem to eliminate methionine as a possible constituent of the insulin molecule. The possible presence of thiolhistidine has also been investigated, with cuprous oxide as a precipitant. This reagent has already been used as a precipitant for such thiol compounds as ergothionine (28) and cysteine (29). Using solutions of synthetic thiolhistidine (30), we have found that this amino acid is apparently quantitatively precipitated by cuprous oxide, while histidine is not.<sup>7</sup> The precipitate obtained by treating a hydrolysate of insulin with cuprous oxide gave none of the color reactions (31) for thiolhistidine. This and similar findings, reported recently by du Vigneaud *et al.* (32), tend to make the presence of this amino acid in the insulin molecule improbable. The possibility must be considered, however, that thiolhistidine if present is destroyed during hydrolysis or the later manipulations of the hydrolysate.

The disadvantage attending the parenteral injection of insulin has induced us to test several of our insulin preparations (Experiments 5, 7, 9, 12, and 13) for activity when given orally. Our finding that they showed no physiological effect on the blood sugar, when administered by stomach tube, is in agreement with similar results of Scott and coworkers (33) who also investigated certain insulin derivatives with the same object.

Since the cystine in the insulin molecule seems to play such a prominent rôle in the physiological action of this hormone, we have tested a number of cystine peptides (diglycylcystine, cystinyldiglycine, and  $\gamma$ -diglutamylcystine) for hypoglycemic action. These substances are, in the doses we have used, inactive. Brand and Sandberg (34) found that dialanylcystine and its anhydride had no action on the blood sugar.

<sup>6</sup> Schoeberl and Eck (27) found that certain dithiol acids decompose under the influence of HCl.

<sup>7</sup> The use of cuprous oxide was suggested to us by Dr. A. White of the Yale Medical School, New Haven. We followed closely the procedure described by Vickery and White for the precipitation of cysteine as the cuprous mercaptide (29).

## SUMMARY

In spite of the remarkably specific physiological action of insulin, chemical investigation emphasizes the typical protein nature of the crystalline hormone. Extensive efforts to isolate a characteristic component responsible for the physiological activity have so far been unsuccessful. Moreover, the isolation in recent years of various enzymes, hormones, and bacterial toxins, and the recognition of many of these substances as characteristic proteins strongly suggest that the specific qualities of these proteins must originate in the manner in which their component amino acids are linked, rather than in the presence of constituents characterized by specific chemical properties. The principal objective of the investigation here reported, definitely to associate the physiological activity of insulin with a localized portion of the molecule, has not been realized. Certainly in most of the reactions we have studied, inactivation is accompanied by a change in either cystine or amino nitrogen content with the single exception of acid alcohol inactivation. This should not imply, however, that no other changes in the molecule can take place under the influence of the different reagents. Whether the changes in cystine and amino nitrogen are confined to a localized portion of the molecule cannot be stated. These quantitative studies, which we hoped would give us an answer to this question, do not permit such an interpretation. It appears, however, from the results here recorded, that the hypoglycemic property of insulin is associated with certain ( $-S-S-$ ) dithio groupings (present, in part at least, as combined cystine) and also with certain free amino groups<sup>8</sup> (present most probably as cystine).

We are indebted to Eli Lilly and Company for their gift of insulin, and to E. R. Squibb and Sons for supplying us with insulin at cost.

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<sup>8</sup> It cannot be decided whether we are dealing with one or several amino groups.

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## SERUM LIPID CHANGES IN RELATION TO THE INTER-MEDIARY METABOLISM OF FAT

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This report is concerned with the investigation of the serum lipids under experimental conditions in which fat of known quality supplied a large proportion of the calories. The experiments were planned to study the influence upon blood lipids (a) of specific dietary fats, (b) of fasting following the period of ingestion of these fats, (c) of a diet poor in fat, and (d) of fasting following the period on the fat-poor diet. Studies of the respiratory quotient indicate that the energy expended by the animal is derived largely from fat when on a high fat diet or during fasting (1, 2). Since the fat burned during fasting is derived from body fat, the character of which is closely related to the previous diet (3, 4), it can be assumed that under these circumstances the animals are actively burning fat of known quality.

### *Material and Methods*

Six healthy adult male dogs were selected and kept under similar conditions during the entire course of the experiments, from January 1, 1935, until April 27, 1935. They were subjected to a preliminary fasting period, partly to deplete their stores of body fat, and were then divided into two groups of three each. One group was refattened on a diet containing coconut oil (iodine number 10; average molecular weight of fatty acids 214), and the other group was refattened on a diet containing linseed oil (iodine number 165; average molecular weight of fatty acids 280). The fat comprised 60 per cent of the calories in each case.

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The diets were composed of casein 21 parts, sucrose 18 parts, Cellu flour 32 parts, fat 27 parts, and salt mixture<sup>1</sup> 2 parts. Because this fatty diet was poorly consumed the non-fat components were fed together after the daily portion of fat was administered by gavage. Cod liver oil concentrate tablets were used as the source of vitamins A and D, while marmite was used to provide vitamins B and G. After a period of 2½ to 3 months, when the animals had regained most of the weight lost during the initial period of fasting, they were subjected to a fasting period of 7 days; blood specimens were collected after 1, 2, 4, and 7 days of fasting. Samples of blood taken 16 to 20 hours after eating had previously been collected during the period on the high fat diet. After the period of fasting following the high fat diet the dogs were offered the ration with molasses substituted equicalorically for the fat component. The body weight increased much more rapidly on this than on the high fat régime and in from 2 to 4 weeks the animals had regained the weight lost during the fasting period. They were again subjected to a fasting period and blood samples were collected at the end of 4 days. During both fasting periods the dogs were given water *ad libitum* and their regular supplement of vitamins.

Their weight changes are shown in Table I. For more ready comparison of the state of nutrition of the different subjects, which varied considerably in size, the nutritive indices were calculated according to the formula used by Cowgill (5) and found to be within the normal range with the exception of Dog 139, which remained overweight throughout the experiment.

Blood specimens were collected from the jugular vein and, after being allowed to clot, were centrifuged to separate the serum. An alcohol-ether extract was prepared according to the method of Bloor (6) with refluxing on the hot-plate for an hour as suggested by Man and Gildea (7). The serum lipids were measured by the microgravimetric technique used by Wilson and Hansen (8), which gives values for the total lipids, saponifiable and unsaponifiable fractions, and the average molecular weights and iodine numbers of the fatty acids. The lipid phosphorus was determined by means of the digestion used by Baumann (9) followed by de-

<sup>1</sup> See Table III on p. 734 of the article by Cowgill (Cowgill, G. R., *J. Biol. Chem.*, **56**, 725 (1923)).

termination of the liberated inorganic phosphorus by the method of Benedict and Theis (10). Total cholesterol and free cholesterol were determined by oxidation of the digitonide precipitate as devised by Okey (11) and modified by Boyd (12). The difference between total and free cholesterol was taken as the value of the cholesterol ester. The iodine number of the phospholipid fatty acids was determined by the method of Sinclair (13). The serum proteins were determined by the usual Kjeldahl procedure on 0.2 cc. of serum (14).

### *Results*

The results of all the determinations are presented in Table I. Since the dogs in each group responded alike throughout, the values at critical points are averaged for the three dogs in each group and presented in graphic form in Figs. 1 to 3. The points chosen may be considered characteristic of serum fat when the dogs are metabolizing fat from different sources; *i.e.*, (a) "high fat diet" at the end of the period of high fat feeding, (b) "fasting 7 days" when the animals had fasted 7 days after the high fat diet, (c) "high carbohydrate diet" at the end of the period on the high carbohydrate regimen, and (d) "fasting 4 days" when the animals had fasted 4 days after receiving a diet high in carbohydrate for several weeks.

In Fig. 1 the chief fractions of the fats are averaged for the three dogs on the diet containing linseed oil and for the three on the diet with coconut oil. The proportionate distribution of the total lipid between the three fractions shows no apparent significant change even with the quite wide variations in the quantity of total lipid. The results are consistent and show that all three fractions have a higher concentration when the animals were fasted 20 hours after a high fat diet; the concentrations are lowest on the high carbohydrate diet, and intermediate values were obtained after both 7 and 4 days fast. The results also show that the lipids are appreciably higher when the animals received a diet which contained a relatively saturated fat (coconut oil) than when the animals received a highly unsaturated fat (linseed oil). In contrast to the decrease in lipids during the fast following the diet high in fat, an increase followed the fast subsequent to the diet high in carbohydrate. However, in both cases the fasting

TABLE I  
Results of Analyses of Serum Lipids

Dog No.	Dog length	Experimental conditions	Fast	Serum protein	Body weight	Unsatifiable fraction				Phospholipid fraction				Saponifiable fraction		Calculated total lipid
						Total	I No.	Cholesterol		Total	Lipid P		I No.	Total	Aver- age mol. wt.	
								mg. per cent	mg. per cent		mg. per cent	mg. per cent				
80	65.8	2½ mos. coconut oil	20	5.59	5.70	245	71.0	216	64	16.25	14.75		542	98	288	903
		3 " " "	20	5.47	5.70	289	66.0		71	17.70	17.00	105	734	96	282	1163
		Fasting	44	5.10	5.40	234	66.0	184	60	12.88	11.12		534	97	281	864
		"	116	5.14	5.10	278	64.0	174	67	15.50	14.50	116	587	108	288	982
		2 wks. high CHO diet	20	5.25	5.88	217	68.0	176	62	13.70	11.50		478	89	287	788
150	60.0	3 " " "	20	5.42	6.34	217	66.0	156	58	13.25	10.25	93	405	91	287	704
		Fasting	92	5.75	5.40	272	64.0	204	68	14.7	13.00	109	542	97	284	905
		2 mos. coconut oil	20	5.62	5.78	238	72.0	209	62	16.85	13.75		525	95	288	872
		2½ " " "	20	5.26	5.90	266	69.0	170	69	16.00	14.70	107	640	93	291	1027
		Fasting	44	5.10	5.50	235	66.0	146	60	12.44	10.50		475	105	284	798
151	75.3	110	5.16	5.00	237	63.0	169	57	12.31	10.75	116	485	108	288	811	
		2 wks. high CHO diet	20	5.62	5.66	177	71.0	139	48	11.80	9.10		390	91	289	641
		3 " " "	20	6.19	6.00	200	65.0	123	57	12.70	10.50	97	375	91	285	657
		Fasting	92	6.07	5.18	210	65.0	165	57	13.75	11.60	118	468	97	302	770
		1 mo. coconut oil	20	5.75	9.24	255	68.0	210	64	16.67	16.10		534	99	284	913
		2 mos. " "	20	5.84	10.38	346	65.0	282	84	22.08	19.45	111	696	98	291	1194
		Fasting	44	5.94	9.82	317	69.0	263	64	20.83	18.75		639	99	294	1100
		"	92	6.51	9.40	347	66.7	265	83	18.95	16.80	123	670	113	296	1151
		"	164	5.84	8.84	278	68.0	171	68	16.88	15.50		584	113	290	985
		2 wks. high CHO diet	20	5.75	10.00	152	69.0	131	41	11.25	9.90	104	349	99	286	578
		3 " " "	20	6.01	10.20	180	68.0	133	49	11.90	11.00		412	96	286	679
		Fasting	92	5.52	9.10	212	62.0		50	12.25	11.75	123	428	107	281	734



values tend to approach a corresponding level. The concentrations of serum protein (Table I) indicate that the changes in concentration of fat were not due to changes in the amount of water in the blood. Figs. 2 and 3 present an analysis of characteristics of the blood lipids during the various changes in the total lipid.

Fig. 2 presents the values of the fatty acids of the serum lipids found combined in the three forms usually encountered in the serum; namely, phospholipid, cholesterol ester, and neutral fat. The fatty acids combined as phospholipid are calculated from the

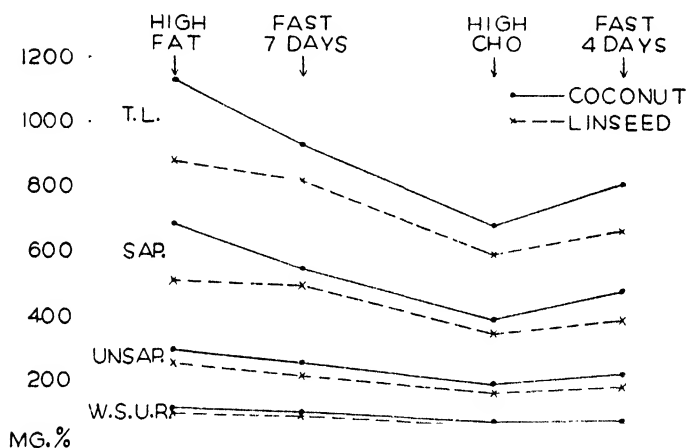


FIG. 1. Changes in the serum content of total lipid (T.L.), saponifiable material (Sap.), unsaponifiable material (Unsap.), and water-soluble unsaponifiable residue (W.S.U.R.) after saponification in the two groups of animals shown at crucial points during the experimental period.

saponifiable phospholipid phosphorus, assuming that each molecule of saponifiable lipid phosphorus combines with 2 molecules of fatty acid.<sup>2</sup> The fatty acids combined with cholesterol are calculated from the content of ester cholesterol.<sup>3</sup> The fatty acids

<sup>2</sup>  $\text{Mg. saponifiable lipid P} \times \frac{\text{molecular weight of fatty acid}}{15.5} = \text{mg. fatty acid present in the phospholipids.}$

<sup>3</sup>  $\frac{\text{Molecular weight of fatty acid}}{386} \times \text{mg. ester cholesterol} = \text{mg. fatty acid present as cholesterol esters.}$

of the neutral fat are determined by difference. It is evident that the fatty acids of both the phospholipid and cholesterol ester fractions behave alike and that the changes in concentration are parallel to those of the total lipid (Fig. 1). The fatty acids of the neutral fat in the dogs fed coconut oil parallel those of the other fractions. However, the fatty acids of the neutral fat of the dogs fed linseed oil tend to remain practically constant throughout the course of the experiment.

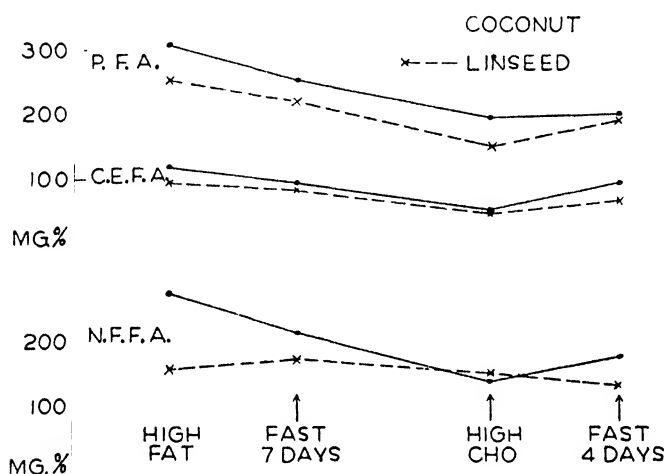


FIG. 2. Changes in serum content of the three principal fractions of the serum fatty acids in the two groups of animals at crucial points during the experimental period. P.F.A. indicates phospholipid fatty acids, C.E.F.A. indicates cholesterol ester fatty acids, and N.F.F.A. indicates fatty acids derived from neutral fat.

In the determination of the qualitative differences of the fatty acids it can be seen from Table I that there is no significant change of the average molecular weight of the total fatty acids. The average number of double bonds per molecule is calculated from the molecular weight and iodine number and varies directly with the iodine number. Fig. 3 presents the determined iodine numbers of the total fatty acids and the phospholipid fatty acids and, in addition, the calculated iodine numbers of the fatty acids of the neutral fat. The latter calculation will be explained later.

It will be noted that the iodine numbers of all fractions in the dogs receiving the coconut oil diet are lower than the corresponding values for the dogs ingesting linseed oil. In contrast to the increase in iodine values during the fast following the feeding of the coconut oil diet, a decrease in iodine values is observed during the fast following the feeding of linseed oil.

On the high carbohydrate diet the iodine numbers of all the fractions decreased, with the exception of the iodine number of

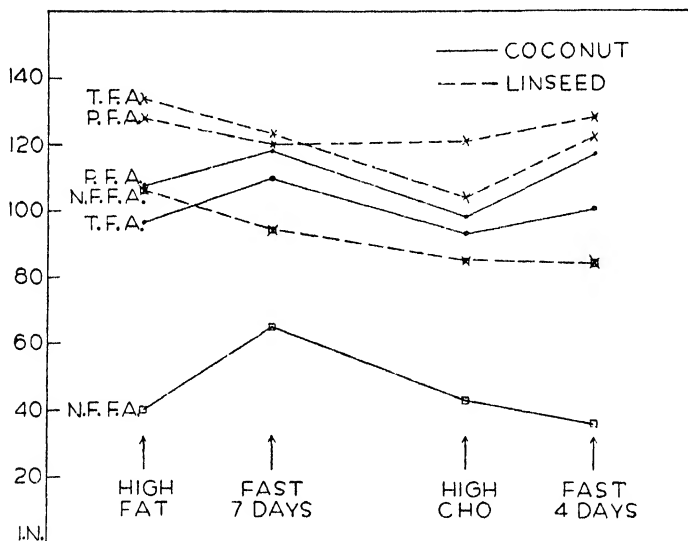


FIG. 3. Average iodine numbers of the fatty acids in the two groups of animals at different stages of the experiments. T.F.A. indicates total fatty acids, P.F.A. indicates phospholipid fatty acids, and N.F.F.A. indicates fatty acids derived from neutral fat.

the phospholipid fatty acids in the animals previously fed the linseed oil diet; in the latter group the values remained practically constant. During the fast following the feeding of the carbohydrate diet the iodine numbers of the total fatty acids and the phospholipid fatty acids rose in all cases. On the other hand the iodine numbers of the fatty acids of the neutral fat decreased. This decrease, however, was not significant in the group originally fed linseed oil.

## DISCUSSION

Since part of the serum lipid may be an integral part of the plasma rather than lipid being transported to various tissues, the changes in the lipids do not necessarily involve the entire lipid portion of the plasma. At present no means is available to distinguish these functionally different lipids. However, since the animals all remained well and in a good nutritive condition, the changes in serum lipid are probably changes in the system of lipid transport. Since all bloods were taken after a fast of at least 16 to 20 hours, the changes reflect the effects of different types of fat metabolism rather than serum lipids as affected by absorption of fat from the gastrointestinal tract (15).

*Quantitative Changes*—There appears to be a direct relationship between the level of serum lipid and the presumable rate at which fat combustion is taking place. Fat was presumably actively burned during the period in which the dogs received 60 per cent of their calories in the form of fat. Concomitantly the serum lipids were high. It has been shown previously (16) that certain animals tend to display an increase in the postabsorptive level of the blood lipids when ingesting large amounts of fat over a long period. After the initial stages of a fasting period the energy exchange of the body is considerably lowered and most of the energy is derived from fat (2). The fat combustion is less under these circumstances than on the high fat diet. On an adequate high carbohydrate diet there is a minimum of fat combustion (1).

Ling (17) reports that the blood fat of dogs decreased on a fat-free diet and dropped further on fasting for 7 days. The data reported in the present paper, while in general agreement with Ling's work, do not agree with the latter finding in that fasting for 4 days after a period on a low fat diet was associated with a rise of serum lipids. Ling's work, however, was done under somewhat different circumstances and the determinations were carried out on whole blood. The metabolic activity of Ling's dogs may have been abnormal as Wesson (18) and Wesson and Burr (19) found in studying the respiratory quotient that rats on a prolonged fat-free regimen had an abnormal metabolism. Ling found that on a fat-free diet the decrease in blood lipids was principally in lipids other than phospholipid and cholesterol.

As illustrated in Fig. 2 the fatty acids combined in all three



forms show changes which parallel the fluctuations in total lipids in the dogs fed coconut oil. However, the fatty acids present as triglyceride did not change significantly in the dogs fed linseed oil. The findings of higher total lipid in the animals fed coconut oil are in agreement with the work of Hansen (20) who found that the level of serum lipids in rats varied inversely with the degree of unsaturation of the dietary fat. Drummond (21) and Bloor (22) have suggested, from the work of Joannovics and Pick (23), that during the absorption of fat the liver selectively removes the unsaturated fatty acids from the plasma in preference to the saturated acids. In the case of linseed oil, which contains large amounts of unsaturated fatty acids as compared to coconut oil, the liver may be removing more of the fat from the blood stream. On the other hand, Leathes and Raper (24) suggest that when depot fat is utilized the fatty acids are mobilized and transported to the liver, when they are desaturated and then transported to the site of their combustion as phospholipid. If desaturation is a necessary preparation for the latter stages of fat combustion, then the unsaturated fatty acids may be utilized more readily than the saturated ones and, therefore, are removed from the blood stream more rapidly.

*Changes in Iodine Number*—Since the body stores and food are the source of serum fats, the finding of a higher iodine number in the dogs fed linseed oil is to be expected. A low iodine number has been previously observed in various species (25, 26), when the animals were fed a diet practically free of fat. The low iodine numbers during the period on the high carbohydrate diet are in keeping with these observations. The tendency of the two groups of animals to have the same iodine numbers at the end of the 7 day fast may possibly be explained by the fact that they had lost most of the weight gained on the special diets. The resulting serum fatty acids would then tend to have an iodine number which is characteristic of stores of fat which are less readily given up. This fat may have similar characteristics in both groups of dogs. Since the period on the high carbohydrate diet is relatively short, the effect of the previous diet may still be reflected in the serum lipids, as indicated by the difference in the iodine numbers in the two groups of animals at this stage.

The greater iodine number (Fig. 3) of the total fatty acids com-

pared with that of the phospholipid fatty acids in animals ingesting linseed oil may be due to the influence of the cholesterol esters, which are known to contain highly unsaturated fatty acids (27, 28). At the end of each of the fasting periods the iodine number of the phospholipid fatty acids in both groups attained approximately the same level; namely, between 115 and 125, though at all stages of the experiments they were higher in the animals fed the linseed oil diet. Apparently the iodine number of the phospholipid fatty acids reflects to some extent the iodine number of the dietary fat, but there is also a tendency for it to assume a level of 110 to 125 after a period of fasting, when presumably there is active burning of the body fat.

Given the iodine numbers of the total fatty acids and those of two of the fractions, the iodine number of the third fraction may be calculated. By assuming a value of 180 for the iodine number of cholesterol fatty acids, the iodine numbers of neutral fat are those shown in Fig. 3. If Schaible's (28) figure of 156 were used, the neutral fat iodine numbers would be given a slightly higher value, but all of the changes would be in a similar direction. It will be seen that the effects of the various experimental conditions are reflected more markedly in the iodine numbers of neutral fat than in those of the phospholipid fatty acids. Reference to Table I, which presents the diet and weight changes of the animals at the different stages of the experiments, shows that the calculated iodine numbers of neutral fat are within a range one might expect to find for the body fat under such conditions (3, 4) and all of the variations occur in expected directions.

Bloor (15) has demonstrated that during the absorption of fat there is a rise in the various lipid constituents of the serum, but the neutral fat is the portion fluctuating most widely and, therefore, must be quite important in the transport of fat either to or from the tissues. According to the calculations mentioned above, the iodine numbers of the body and dietary fat are much more closely reflected in the fatty acids derived from neutral fat than from either of the other two fractions. This evidence, together with the fact that the body depots are almost exclusively neutral fat (4), suggests that the fat is first mobilized from the body stores and carried in the serum as neutral fat.

The quantitative changes in the phospholipids indicate that

they enter into lipid transport in a manner similar to the neutral fat. However, the qualitative changes in the phospholipid fatty acids are much less marked and are influenced to a much smaller extent by diet and the stress of increased metabolism. It has been suggested (24) that phospholipid may be derived from the food or depot fat after partial preparation for combustion has occurred in the liver. Another factor is the probability that part of the phospholipid does not enter into the process of lipid transport but is present as an integral part of the plasma. These influences would tend to diminish or mask qualitative changes in the phospholipid to a greater extent than they would affect the quantitative changes. This would seem to imply that while the neutral fat may be concerned solely with transport, the phospholipid is present as a tissue constituent of the plasma as well as an agent in the transport of fatty acids.

While it is difficult to make a detailed interpretation of the data presented in the present study, certain findings are quite consistent. The total quantity of the blood lipids seems to vary with the amount of fat combustion taking place in the body. During the process all lipids increase, which suggests that they all play a part in the utilization of fat. The data indicate that the neutral fat is especially concerned with the transport of fat, both to the depots for storage and from the depots to the site of utilization. The data further indicate that, although the phospholipid and cholesterol may be partly an integral part of serum, they are likewise concerned with transport and are in some manner acting as agents in the ultimate utilization of fat.

#### SUMMARY

In order to deplete their fat depots, six dogs were fasted until they had lost approximately 25 per cent of their body weight. The nutritional state of the animals was restored with a diet which was complete in all essential nutrients and which furnished 60 per cent of the calories as fat. Three of the dogs received linseed oil as the source of fat, while the remaining three received coconut oil. When the animals had regained most of the weight previously lost, they were again fasted, this time for a period of 7 days. All the dogs were then fed a diet in which carbohydrate was substituted for the fat. This was followed by a fast of 4 days.

Serum samples were obtained at various intervals for analysis of the various lipid fractions. The following observations were noted.

1. The highest values for the total lipids are obtained during the ingestion of fat and the lowest during the ingestion of carbohydrate with intermediate, and almost equal, values at the end of the two fasting periods. The level of the serum lipid appears to vary with the intensity of fat metabolism.

2. All of the principal fractions of the total lipid take part in the fluctuations to approximately an equal extent, indicating that all are concerned with the transport and utilization of fat.

3. The dogs ingesting coconut oil have higher serum lipid values throughout than those receiving linseed oil. The difference is most marked in the neutral fat.

4. The iodine numbers of the total fatty acids and, to a lesser extent, those of the phospholipid fatty acids, reflect the character of the diet during the feeding periods and the character of the depot fat during fasting. This effect is much more strikingly shown in the calculated iodine numbers of the fatty acids derived from neutral fat than in the iodine numbers of the other fatty acids.

5. Since the iodine number of the fat being transported in the plasma, whether derived from food or body depots, is much more closely reflected in the neutral fat than in the other fractions of the serum lipids, it is suggested that the neutral fat functions primarily as a transport agent. On the other hand the phospholipid and cholesterol ester fractions, while aiding in the transport of lipids, appear to be concerned more with the ultimate combustion of fat.

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## THE EXTRACTION OF BLOOD LIPIDS\*

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What is now generally known as the Bloor extract is an alcohol-ether solution of lipids from blood or tissues prepared by extracting with relatively large volumes of solvent and a few minutes of heating. The procedure is simple and convenient and has for the most part replaced earlier methods in which lipids were extracted with small volumes of solvent and long heating or by repeated shaking and extraction in separatory funnels. From time to time occasional criticisms of the method have appeared, but these have in general been satisfactorily explained. Recently, however, the essential simplicity of the method has been abandoned in a number of studies, for example those of Man and Gildea (1932-33), Man and Peters (1933), Kirk, Page, and Van Slyke (1934), Page, Kirk, Lewis, Thompson, and Van Slyke (1935), and Wilson and Hansen (1935-36). The modification adopted in all of these studies was that of Man and Gildea (1932-33) and consisted in refluxing the Bloor extract for 1 hour.

Unfortunately this detracts considerably from the convenience of the Bloor technique. It requires more time and more apparatus, which in intensive studies may require much reduplication. Of more importance is whether or not the modification is essential, and may the Bloor method be more simply modified to meet the criticism of supposedly incomplete extraction? Man and Gildea (1932-33) stated that, "In eight experiments 5.0 to 31.0 per cent greater quantities of fatty acid were recovered when plasma was refluxed 1 hour with alcohol-ether mixture than when aliquots of the same plasma were extracted by Bloor's method." Kirk,

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Page, and Van Slyke (1934) compared the determination of total lipid and cholesterol by the two methods in two samples of plasma and, referring to their results, Page *et al.* (1935) stated they "found no significant effect of this difference in mode of extraction on values obtained for either free or total cholesterol." Stewart and Hendry (1935) found that boiling the Bloor extract did not increase the titratable fatty acids when a stream of hydrogen was kept passing through the extract, suggesting that the apparently increased extraction of fatty acids by heat is really an artifact due to introduction of acidic groups at double bonds.

It would appear that in the hands of some investigators the Bloor method can be used satisfactorily for extracting all lipids capable of being extracted with alcohol-ether, while in the hands of others it cannot. The reason for this would seem to be that the limits in the application of the Bloor method have not been sufficiently defined. The method was described by Bloor in two papers appearing in 1914 and 1915. The procedure given was to run 3 cc. of blood slowly into 70 to 80 cc. of alcohol-ether (3:1, both freshly redistilled) with shaking, heat to boiling, cool, make up to 100 cc., and filter. It was stated (Bloor, 1915) that, "The solvent combines the penetrating power of alcohol with the greater solvent power of ether. Under these conditions the short heating is adequate to extract all but the most difficultly extractable fatty material from the whole blood and . . . to extract the lipoids completely from serum or plasma. Moreover, . . . gentle treatment is believed not to decompose the phosphatides appreciably."

The original technique was varied several times in the subsequent reports of Bloor and his associates. In 1922 (Bloor, Pelkan, and Allen) a dilution of 5 cc. of blood in 100 of alcohol-ether was used; in 1928 (Bloor) dilutions from 1:16.7 to 1:50 were employed; in 1932 (Bloor) there appeared a dilution as low as 1:12.5. In 1929 Bloor stipulated the use of 95 per cent alcohol instead of the alcohol-ether mixture for whole blood and in addition introduced the necessity of peroxide-free ether.

It appeared that information was needed on a number of points in this method of extraction in which the procedure might be expected to vary in the hands of different investigators, where-with the method might be improved and enlarged and whereby the limitations of the method might be established. With this

end in view the experiments herein reported were begun. Extracts of blood lipids were prepared in various ways but all were analyzed by the author's modification of the oxidative micro-technique (Boyd, 1933).

(a) *Dilution of Serum or Plasma Extracts*—The first point investigated was whether or not the completeness of extraction is influenced by the extent to which plasma or serum is diluted in alcohol-ether in preparation of an extract by Bloor's method. A series of extracts was made from a composite sample of human sera, 3 cc. of which were added slowly and with shaking to 15, 30, 60, 90, 120, and 150 cc. of alcohol-ether (3:1, both freshly redistilled). This gave dilutions varying between 1:5 and 1:50. The extracts were heated to boiling with frequent shaking to prevent superheating and the total heating time was 5 minutes in each case. When cool, the extracts were filtered, the precipitate washed, and the combined filtrate made up to volume.

72 analyses were performed on different samples of sera. The results from a typical experiment have been plotted in Fig. 1. It was found that a dilution of 1:20 was the minimum which gave a maximum extraction of all lipids with this solvent. Dilutions greater than this did not increase the yield of extracted lipids, but dilutions less than 1:20 gave incomplete extraction. It will be noted that the dilution of 1:20 was the initial dilution of the solvent. Some of the solvent is necessarily lost in heating, but more is added with the washings, so that the final volume was greater than 1:20. To allow for this and also to give a margin of safety, the technique adopted from this experiment was to add 3 cc. of serum or plasma to about 80 cc. of alcohol-ether and make the final volume to 100 cc. It is of interest that this was the dilution used by Bloor (1914, 1915) in his original work.

The results also demonstrated that some lipids are more readily extracted than others. Phospholipid and free cholesterol were extracted almost as readily with the smaller volumes as with the larger, but with smaller volumes the extraction of neutral fat and cholesterol esters was much more incomplete. It may be concluded that neutral fat and cholesterol esters are less readily extracted than phospholipid and free cholesterol.

(b) *Dilution of Whole Blood Extracts*—Since there is an unequal distribution of lipids between plasma and red blood cells, plasma



or serum analyses are preferable to whole blood. Nevertheless, whole blood continues to be used. It has been implied in several studies that whole blood is more difficult to extract than plasma or serum. Hence a series of extracts of whole blood was made with varying dilutions of solvent, as in the previous experiment. The phospholipid and free cholesterol only of these extracts were analyzed, since the red blood cells contain chiefly these two groups of lipids and very little cholesterol ester and neutral fat.

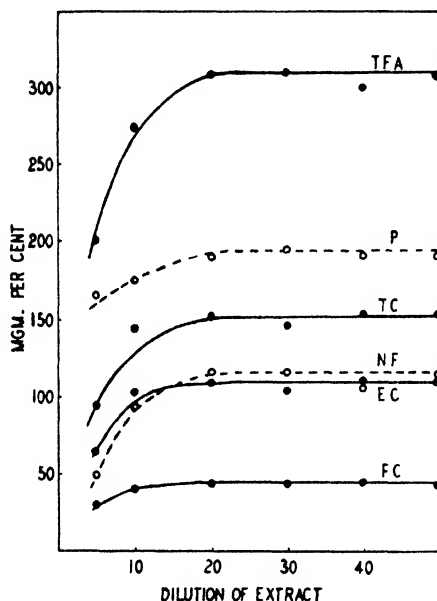


FIG. 1. The effect of dilution in alcohol-ether on the extraction of serum lipids. *TFA* = total fatty acids; *P* = phospholipids; *TC* = total cholesterol; *NF* = neutral fat; *EC* = ester cholesterol; *FC* = free cholesterol.

92 analyses were performed on several composite samples of human blood and the results have been recorded in Fig. 2. Whole blood was found to be more difficult to extract than serum. Complete extraction of phospholipid required a dilution of 1:30 to 1:35, and about the same minimum values held for free cholesterol, but there was a better extraction of this lipid with the lower dilutions.

(c) *Use of Heat in Extraction*—The next factor studied was the

effect of heating extracts which had been sufficiently diluted to give maximum extraction with the few minutes of heating used in the Bloor method. For this purpose, 3 cc. of mixed human sera were added to 90 cc. of alcohol-ether, one extract left un-

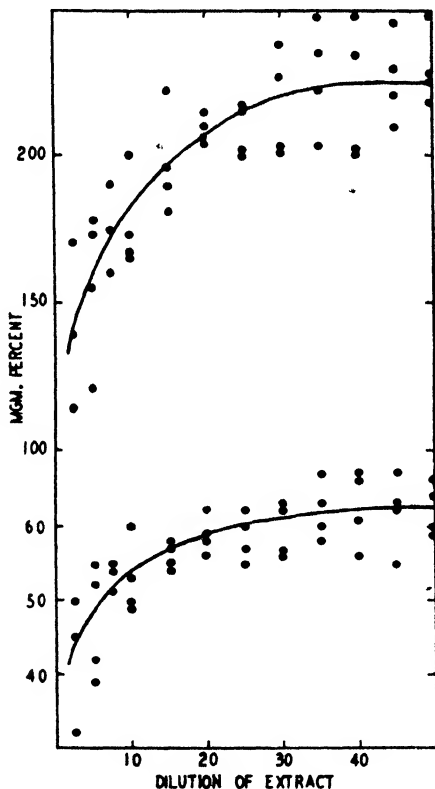


FIG. 2. The effect of dilution in alcohol-ether on the extraction of phospholipid (upper curve) and free cholesterol (lower curve) from whole blood.

heated, another heated 5 minutes (the usual time in the Bloor technique), and the remaining heated 15, 30, 60, and 120 minutes respectively. The solvent lost by evaporation was replaced with fresh alcohol-ether every 15 minutes.

72 analyses were performed on different samples of sera, and

the results from a typical experiment are reported in Table I. It was found that boiling the extract from 5 minutes to 2 hours had no appreciable effect on the yield of extracted lipids. In fact, just as high values were obtained without any heat at all. These results do not necessarily contradict the findings of Man and Gildea (1932-33), because presumably they used smaller dilutions, which have been shown to give incomplete extraction which conceivably might have been remedied by the use of an hour's heating.

Heat did not affect the color of serum extracts but caused rapid coloration of whole blood extracts, which became a dark, reddish brown. Unfortunately much of this colored material, which is

TABLE I

*Effect of Varying Periods of Heat on Extraction of Serum Lipids Diluted Over 25 Times in Alcohol-Ether*

The results are expressed in mg. per 100 cc. of serum.

Heating time.....	None	5 min.	15 min.	30 min.	1 hr.	2 hrs.
Total lipid.....	620	662	628	597	608	650
Neutral fat.....	159	162	163	145	148	175
Total fatty acids.....	374	397	385	355	362	397
Total cholesterol.....	170	185	160	172	176	175
Ester ".....	129	147	118	137	143	135
Free ".....	41	38	42	35	33	40
Phospholipid.....	205	216	226	188	188	209

presumably a decomposition product of hemoglobin, has solubilities similar to those of lipids. It precipitated in part with cholesterol digitonide and with the acetone precipitate of the phospholipids, but analyses revealed that it affected chiefly the calculated values for neutral fat. These became progressively greater the longer the heating time and after 1 hour's boiling they were 3 times as high as in the unheated extract. Since the value of serum neutral fat is not increased by heat under the same circumstances and since the red blood cells could not possibly contain enough neutral fat to account for the marked increase apparent in the calculated values, it was concluded that the increase was due to contamination with this colored material. Neutral fat is the term applied by the author to residual fatty acids not ac-

counted for in cholesterol esters and phospholipid after these have been subtracted from the total fatty acids. Thus values for neutral fat would be increased if the total fatty acid values were raised by solution of much of this colored matter in petroleum ether. Actually, the petroleum ether extract of the saponified material was highly colored when heat had been used in making the original alcohol-ether extract. It was impossible to tell whether heating increased the extraction of whole blood lipids, but since heating introduced foreign material, it obviously should not be used under these circumstances.

(d) *Cold Extraction of Serum or Plasma Lipids*—Since the unheated extract of serum in the previous experiment contained just as much lipid as the heated ones, it was thought possible that prolonged periods of cold extraction might increase the yield of extracted lipids. This was investigated by preparing extracts of 3 cc. of mixed human sera as before and allowing these to stand for varying periods of time in a dark place, tightly stoppered and occasionally shaken. 72 estimations were made on different samples of sera. It was found that extracts which had stood for 0 hours (actually less than 15 minutes) contained just as much lipid as those which had stood for longer periods up to 2 days. This experiment indicated that serum, and presumably also plasma, is quickly extracted if sufficiently diluted with cold alcohol-ether.

(e) *Does Heat Destroy Serum or Plasma Lipids Extracted by Alcohol-Ether?*—Under (c) it was found that heat did not increase the yield of extracted lipids. It was possible that heat did increase the extraction of lipids but at the same time decomposed some, the net effect being no apparent difference. This was investigated by preparing four extracts of 3 cc. of human sera as before, allowing them to stand 18 hours without heat, filtering, and subjecting half of the filtrates to an hour's boiling. Heat was found to have no destructive effect on the lipids contained in sufficiently diluted alcohol-ether extracts of serum, except to produce a very slight decomposition of cholesterol esters. This latter was not marked, but appeared in nearly all of the experiments done. Phospholipids were not destroyed by an hour's boiling; Bloor (1915) suggested that phospholipids might be decomposed by heat but it is likely that he had in mind the prolonged (12 to 24 hours) heating

in use at that time. Since an hour's boiling did not destroy lipids, it is obvious that the same amount of heat does not enhance the extraction of lipids in sufficiently diluted extracts of serum.

(f) *Percentage of Alcohol-Ether in Extracting Fluid*—It was felt that some of the discrepancies in the results of those who have used the Bloor method of extraction might have been due to variations in the relative percentages of alcohol-ether used in the extracting fluid. Hence 3 cc. of mixed human sera were added to 80 cc. of each of the following: 95 per cent alcohol alone, a mixture of 3 parts of alcohol to 1 part of ether, a mixture of 1 part of alcohol to 1 part of ether, and a mixture of 1 part of alcohol to 3 parts of ether. In the first three instances the usual fine precipitate resulted, but in the fluid containing 75 per cent of ether the serum fell to the bottom and coagulated in a mass which was fairly easily broken up with a glass rod.

64 analyses were performed on different samples of sera. It was found that each of the extracting fluids used dissolved about the same amount of lipids. Hence varying the percentages of alcohol and ether could not account for any major differences in the results of different investigators.

(g) *Amount of Lipid Extracted*—Among the many recent micro-methods proposed for lipid analysis, the range of amounts estimated varies considerably. Whether or not small and large aliquots of serum or plasma are equally well extracted has not been studied. To investigate this dilution curves were obtained as under (a) for varying aliquots of the same sample of mixed human sera. In order to keep all other factors the same, the procedure adopted was to dilute the serum with normal saline and then use the same number of cc. of the diluted and undiluted serum in preparing the extracts. Thus progressively smaller amounts of lipid were being extracted from the same volume of material. Analysis of the resulting extracts was confined to the estimations of phospholipid and free cholesterol.

In describing the rather extensive data obtained, it will be necessary merely to give the minimum dilution of extract which was found to give maximum extraction of lipids at the varying dilutions of serum in a typical case. It required a minimal dilution of 1:50 to extract all the phospholipid and free cholesterol from 0.25 cc. of serum plus 1.75 cc. of saline; 1:40 for 0.50 cc.

of serum plus 1.50 cc. of saline; 1:30 for 1.00 cc. of serum plus 1.00 cc. of saline; and 1:20 for 2.00 cc. of serum alone. Hence as progressively smaller amounts of lipid were being extracted, progressively greater dilutions of the extract were required to give maximum extraction. It may be concluded that small amounts of lipid are more difficult to extract than larger amounts within the range and under the conditions herein studied.

In addition to this the results showed that the calculated value for serum lipids varied according to the aliquot of serum used in analysis when this aliquot became lower than a certain minimum. In Table II have been shown the mg. of phospholipid and free cholesterol found by analysis in the optimal dilutions of the aliquots of serum used, together with the calculated values for

TABLE II  
*Oxidative Microestimation of Phospholipid and Free Cholesterol in Varying Aliquots of the Same Sample of Serum*

Aliquot analyzed	Phospholipid		Free cholesterol	
	mg.	mg per cent	mg.	mg. per cent
0.25 cc. + 1.75 cc. saline	0.41	164	0.18	72
0.50 " + 1.50 " "	0.90	180	0.30	60
1.00 " + 1.00 " "	1.92	192	0.52	52
2.00 " + 0.00 " "	3.84	192	0.98	49

the serum lipids. It was found that when less than 2 mg. of phospholipid were estimated, the calculated value for serum phospholipid fell. The converse was true for cholesterol; when less than 0.5 mg. was analyzed, the calculated serum values became progressively higher. Very small aliquots of plasma were used by Page, Kirk, Lewis, Thompson, and Van Slyke (1935) and these authors got lower values for phospholipid and higher values for cholesterol (see Table III) than most other investigators who used approximately the same amounts employed by the author. It would seem probable that the small aliquot accounted for this.

#### DISCUSSION

It has been shown that the failure of several recent investigators to obtain satisfactory results with the Bloor diluted alcohol-

ether extract of blood lipids was due probably to a previous lack of knowledge concerning the limitations of the method and the factors which affect extraction. In the first place it was found that complete extraction could not be obtained with less than 20 volumes of solvent in the case of serum or plasma or with less than 30 volumes in the case of whole blood. When such sufficiently diluted extracts were made, periods of boiling up to 2 hours did not increase the yield of extracted lipids. Since this amount of heat did not destroy the dissolved lipids, it was concluded that neither did it cause any further extraction of lipids. In fact, serum lipids were found to be readily dissolved if sufficiently diluted in cold alcohol-ether and periods of cold extraction up to 2 days did not further the yield over periods of less than 15 minutes. Furthermore, heating is decidedly undesirable in the extraction of whole blood, because it causes the solution of colored decomposition products, probably of hemoglobin, which produce apparent higher values for all lipids and especially for neutral fat.

Variations in the percentages of alcohol and of ether in the extracting fluid over a wide range did not appreciably affect the extraction of blood lipids. On the other hand, aliquots of serum smaller than 2 to 3 cc. require relatively greater dilutions of alcohol-ether to produce complete extraction. When these smaller aliquots are used, the value calculated for serum falls in the case of phospholipid and rises in the case of cholesterol. These differences in calculated values would appear to be due to unknown factors in the acetone precipitation of the phospholipids, on the one hand, and in the digitonin precipitation of the cholesterol, on the other.

These several factors undoubtedly have affected many of the published values for normal human blood lipids. In the case of plasma there is also another factor, that of the anticoagulant used. Schmidt (1935) found phospholipids approximately 13 per cent higher in heparinized than in oxalated plasma; Sperry and Schoenheimer (1935) noted the cholesterol fractions to be about 15 per cent higher; Kirk, Page, and Van Slyke (1934) found total fatty acids some 13 per cent higher; and Man and Gildea (1932-33) reported them 3 to 8 per cent higher. The author has investigated the effect of oxalate on the complete lipid composition of human plasma. Samples of blood were taken from six persons,

half of it oxalated and half defibrinated in order to eliminate entirely the effect of any anticoagulant or of the contraction of the clot in the case of serum. Practically all of the results indicated that oxalated plasma had lower values than defibrinated, but there was considerable variation in the extent of this, which is being subjected to further study.

Page, Kirk, Lewis, Thompson, and Van Slyke (1935) have collected together a number of recently published values for normal human plasma lipids. Unfortunately some of the authors quoted in their list used oxalated plasma and some heparinized plasma or serum. Hence a similar tabulation has been prepared

TABLE III

*Lipid Composition of Oxalated Human Plasma As Calculated from Published Mean Values of Several Recent Investigators*

The results are expressed in mg. per 100 cc. of plasma.

Authors	Total lipid	Composition of total lipid					
		Neu- tral fat	Total fatty acids	Cholesterol			Phos- pho- lipid
				Total	Ester	Free	
Gardner and Gainsborough (1927)				153	99	54	
Boyd (1933)	589	154	353	162	115	47	196
Wilson and Hansen (1935-36)	591		326	164			189
Boyd (1935)	595	150	349	177	124	53	185
“ (1934)	617	154	362	181	128	53	195
Man and Peters (1933)			358	186			189
Page <i>et al.</i> (1935)	661	202	391	209	135	74	163

in which this and other factors have been corrected for and to which one or two other recently published figures have been added, and these data are presented in Table III. A lump value of 10 per cent was deducted from values originally reported for heparinized plasma, milli-equivalent weights per liter have been changed to mg. per 100 cc., and other minor calculations made to bring all to as nearly the same common denominator as possible. Only mean values have been reported; the average standard deviations varied between about 10 and 25 per cent of the means, except in the case of those of Page *et al.* in which it was somewhat higher. In spite of these needed calculations and the fact that the values were obtained by different investigators using different



methods there is comparatively little variation between most of the values.

#### SUMMARY

It was found that when alcohol-ether extracts of blood are sufficiently diluted, lipids are rapidly and completely extracted. Heat or prolonged periods of cold extraction do not increase the yield of lipids. An hour's boiling does not destroy extracted lipids but causes the solution of colored products from whole blood, which results in giving false high values, especially for neutral fat. Lipids are extracted equally well by proportions of alcohol and ether varying over an extensive range. Very small amounts of lipids are more difficult to extract; too small aliquots of serum give low values for phospholipid and high values for cholesterol. Defibrinated plasma contains more of all lipids than oxalated plasma.

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# A COMPARISON BETWEEN THE BENEDICT-DENIS AND PARR BOMB METHODS FOR THE DETERMINATION OF TOTAL SULFUR IN PLANTS AND PROTEINS\*

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Owing to the important rôle of sulfur compounds in biological processes, the biochemist needs a rapid and accurate method for the determination of organic sulfur in biological material. Many procedures for the determination of sulfur in organic compounds have appeared. In nearly all the sulfur is precipitated as barium sulfate but the methods of oxidation vary.

The oxidation method of Benedict (2) as modified by Denis (5), designed for the determination of total sulfur in urine, was applied to other substances by Hoffman and Gortner (7). They found that the method gave satisfactory results when applied to a large number of organic compounds, including proteins. Frear (6) applied the method to foodstuffs, *i.e.* corn, buckwheat, cabbage, spinach, and onions, and the results he obtained were in good agreement with those obtained by the official sodium peroxide fusion method (8). At the present time many investigators are using the Benedict-Denis method.

In the course of an investigation in this laboratory the total sulfur was determined in a large number of cereals and cereal proteins. The procedure followed was that described by Hoffman and Gortner (7) and Frear (6), with the Benedict-Denis method of oxidation. 3 gm. of cereal or 1 gm. of protein was oxidized. With 3 gm. samples, 50 cc. or more of the oxidizing solution were used. Frear in a study of the Benedict-Denis procedure indicated that

\* Published with the permission of the director of the South Dakota Agricultural Experiment Station as communication No. 20 from the Department of Experiment Station Chemistry.

TABLE I  
Sulfur Content of Cereals and Proteins

	Description	Sample No.	Benedict-Denis method*	Parr bomb†	Difference	Benedict-Denis Parr bomb × 100
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Cereals‡	Wheat	582	0.174	0.211	0.037	82.5
	"	583	0.203	0.227	0.024	89.4
	"	459	0.200	0.221	0.021	90.5
	" flour	712	0.159	0.170	0.011	93.5
	Middlings	714	0.223	0.289	0.063	77.2
	Bran	715	0.203	0.267	0.064	76.0
	Corn	570	0.134	0.141	0.007	95.0
	Barley	586	0.129	0.145	0.016	89.0
	"	608	0.136	0.173	0.037	78.6
Proteins§	Gluten	582	0.630	0.740	0.110	85.1
	"	459	0.525	0.700	0.175	75.0
	"	607	0.464	0.644	0.180	72.0
	"	6	0.622	0.700	0.078	88.9
	Gliadin	12	0.238	0.574	0.336	41.5
	"	24	0.267	0.474	0.207	56.3
	"	17	0.666	0.741	0.075	89.9
	Glutenin	11	0.601	0.770	0.169	78.1
	"	18	0.530	0.712	0.182	74.4
	"	23	0.470	0.590	0.120	79.7
	Corn protein	21	0.796	1.011	0.215	78.7
	Commercial casein		0.358	0.644	0.286	55.6
	Purified casein		0.295	0.651	0.356	45.3

\* Probable error of values by Benedict-Denis method,  $\pm 0.011$ .

† Probable error of values by Parr bomb method,  $\pm 0.010$ .

‡ These values are higher than those usually reported for the cereals. This was due to the fact that the cereals were grown in a semiarid region, so contained more protein than cereals grown under more favorable growing conditions.

§ Most of the samples were crude preparations representing a class of proteins with percentages of N between 13 and 15.

|| This sample and the following six were obtained from the same lot of wheat.

this quantity of oxidizing reagent was sufficient. Consistent results were difficult to obtain on duplicate samples, varying frequently by several mg. It soon became evident that the re-

sults obtained on proteins were low, so comparisons were made by the Parr bomb method (9). 0.5 gm. samples were oxidized in the Parr bomb. The ash from two oxidations was combined before precipitating the sulfate ion when determining sulfur in the cereals. Duplicate determinations by the Parr bomb method were very close. Owing to the popularity of the former method, a comparison of the results obtained by the two methods on cereals and proteins is shown in Table I.

The values given represent two or more determinations. In nearly all cases the weights of barium sulfate were within 2 mg. before they were averaged. A large number of determinations by the Benedict-Denis method were discarded. Although there is little difference in the probable error of the values given, much larger samples were used in the Benedict-Denis procedure.

It is evident that the Benedict-Denis method gives low results. These low results are increasingly apparent in the protein samples, as will be noted in Table I.

Since cystine and methionine are the two known organic sulfur compounds in the above material, it must be that some sulfur in one or both of these compounds escapes oxidation. Total sulfur was determined in two protein hydrolysates which were obtained by hydrolyzing protein by alkaline solution in the presence of plumbite to remove the labile (most of the cystine) sulfur. The following results were obtained.

Hydrolysate No.	Percentage of sulfur			
	Benedict-Denis	Parr bomb	Difference	$\frac{\text{Benedict-Denis}}{\text{Parr bomb}} \times 100$
1	0.379	0.427	0.048	88.8
2	0.232	0.336	0.104	69.0

It is apparent that some sulfur besides cystine sulfur fails to be oxidized to sulfate by the Benedict-Denis reagent. Hoffman and Gortner (7) found that this reagent would quantitatively oxidize cystine sulfur to sulfate. Evidently methionine was the compound whose sulfur was not oxidized to sulfate. Table II shows the results obtained on methionine and cystine samples by the Benedict-Denis, Parr bomb, and official sodium peroxide fusion methods.

These results show conclusively that determinations of organic sulfur by the Benedict-Denis reagent where methionine occurs are in error. Since Baernstein (1) has shown that nearly all plants and proteins contain methionine in appreciable quantities, the application of the Benedict-Denis method to oxidize sulfur in these materials is precluded. No other sulfur compounds were subjected to the two methods, although Blumenthal and Clarke (4) give evidence that other sulfur compounds are present in certain proteins.

TABLE II  
*Per Cent Sulfur Obtained by Different Methods*

Material	Benedict-Denis	Parr bomb	Sodium peroxide	Calculated S	$\frac{\text{Benedict-Denis}}{\text{Parr bomb}} \times 100$
Cystine* . . . . .	25.50	26.30	26.24	26.69	97.0
“† . . . . .	25.39	26.24	26.22	26.69	96.8
“‡ . . . . .	25.21	26.35	26.30	26.69	95.7
Methionine§. . . . .	7.80	20.71	20.33	21.50	37.7
“   . . . . .	7.70	20.44	20.17	21.50	37.7
“¶ . . . . .	7.28	20.73	20.55	21.50	35.1

\* Eimer and Amend.

† Kindly supplied by Dr. R. A. Gortner.

‡ Pfanstiehl Chemical Company.

§ Eastman Kodak Company.

|| Kindly supplied by Dr. V. du Vigneaud.

¶ Chemistry Department, University of Illinois.

An interesting observation was made when comparing the differences in sulfur contents obtained by the two methods. From Table I the greatest difference in the percentage of total sulfur obtained by the two methods was in the gliadin Samples 12 and 24, and the two caseins. Samples 12 and 24 were gliadins obtained by dispersing crude wheat gluten in 0.4 per cent sodium hydroxide in approximately 70 per cent ethyl alcohol and precipitating the glutenin by neutralizing the solution. This procedure affected not only the sulfur determinations, but also the total sulfur. Bertrand and Silberstein (3) reported sulfur values as low as 0.349 per cent in gliadins extracted from crude gluten by alcohol containing a little alkali. It appears that the alkaline alcoholic solutions have a

very destructive action on a sulfur compound in the proteins. If some of the sulfur was split from the cystine, the low results obtained by the Benedict-Denis method might be explained by the greater proportion of methionine sulfur remaining in the protein. In case there was some sulfide sulfur present, very little would be oxidized by the Benedict-Denis reagent, because when this reagent is used to oxidize sulfide sulfur (as PbS) a very poor recovery of sulfur as sulfate is obtained. The low sulfur value obtained by the Benedict-Denis method of oxidation of casein would be expected from Baernstein's tables. He gives values of 84.2 and 90.2 per cent of the total sulfur in two caseins as methionine sulfur.

It should be noted that the sulfur contents of the gliadins and glutenins differ greatly, although they were from the same source. Different methods of separating the proteins were used. The differences observed could not be accounted for by the nitrogen contents.

#### SUMMARY

Total sulfur has been determined by the Benedict-Denis and Parr bomb methods on a number of cereals and proteins. The Parr bomb method gave higher values with both the cereal and protein samples. Three cystine and three methionine samples were compared by these and the official sodium peroxide fusion method. An average of 96.5 per cent of the cystine sulfur was recovered by the Benedict-Denis method, but only 36.8 per cent of the methionine sulfur was recovered. The results of the peroxide fusion method agreed well with those of the Parr bomb method. The low sulfur values in two of the proteins suggest that the alkaline alcoholic solutions have a very destructive action on a sulfur linkage in proteins.

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## PURIFICATION OF THE ANTIIHEMORRHAGIC VITAMIN

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The existence of a new fat-soluble vitamin required by chickens to prevent a hemorrhagic disease in which the blood fails to clot has been proved by Dam (1) and by Almquist and Stokstad (2). It is the purpose of the present communication to describe progress in the isolation of this vitamin and to present the details of a rapid method by which the vitamin may be obtained in highly concentrated form.

Assays were conducted on various preparations by adding them to a basal diet composed of ether-extracted fish meal 17.5 parts, ether-extracted brewers' yeast 7.5 parts, ground polished rice 73 parts, salt plus small amounts of ferrous and cupric sulfates 1 part, and cod liver oil 1 part. When given only this basal diet, chicks developed the hemorrhagic deficiency disease within 2 weeks and usually all died of the disease within 4 weeks. Assays were made by using the unsupplemented basal diet with one group of chicks and adding various levels of the preparations to the basal diet fed to other groups from the same lot of chicks. Chicks were examined at 3 day intervals for symptoms of the hemorrhagic disease, as described in preceding papers (2).

The most potent practical source of this vitamin yet discovered is alfalfa meal ((2) p. 105). We have found by means of assays with chicks that the vitamin may be rapidly removed from alfalfa meal by continuous extraction with hexane. This hydrocarbon solvent extracts less extraneous material than do other types of solvents.

In the early stages of the work, the extracted material was saponified in alcoholic potassium hydroxide to remove fat and chlorophyll. It was soon noticed that the non-saponified frac-



tion had up to 50 per cent less potency than the crude extract, depending upon the care and amount of alkali used in saponification. Further treatment of a non-saponifiable preparation with alkali in carefully refined ethyl alcohol caused a loss of more than 75 per cent of its activity, showing that the vitamin is alkali-labile and that the destructive process involves more than a mere racemization.

To avoid this saponification step, efforts were directed toward finding an adsorption reagent for the removal of the green pigments. Activated magnesium oxide was found suitable for this purpose. When added to the hexane extract in amount just sufficient to remove the green coloration, activated magnesium oxide caused no perceptible loss of the antihemorrhagic vitamin. The magnesium oxide sludge was readily removed by centrifuging or by filtration. This procedure was far more convenient and rapid than saponification.

After the magnesium oxide treatment, activated carbon was added to the extract for the removal in part of the remaining red and orange pigments, carotene and xanthophyll. The addition of activated carbon was performed slowly and with continuous stirring up to the point at which the fine carbon particles coalesced and settled rapidly when stirring was stopped. The carbon was removed by filtration and washed with hexane. Under these conditions, there was no appreciable loss of the antihemorrhagic vitamin, although the greater part of the xanthophyll was removed.

The third purification step consisted of concentrating the hexane solution and chilling out the fats and sterols. For example, the extract from 6 kilos of alfalfa meal, after the purification by adsorption, was concentrated under a vacuum to 200 cc., and placed in a refrigerator at  $-1.1^{\circ}$  for 24 hours. A large bulk of solid material separated and was removed by centrifuging. The solids were redissolved in a minimum quantity of warm hexane, chilled out, and centrifuged once more. The second filtrate was combined with the first and concentrated to 100 cc. The chilling out procedure was repeated as before. It was found that the material removed at this stage was inactive even at high dosages, when further purified by crystallization from hexane. For practical purposes, only one additional chilling out was required to conserve

the vitamin in the hexane filtrate. After these concentration and chilling out stages, the hexane filtrate had an orange color.

The fourth purification step involved partition between hexane and 90 per cent methyl alcohol saturated with hexane. The hexane solution was placed in an upright glass column into which was dropped the methyl alcohol-water-hexane mixture. The height of the drop was regulated so as to cause the extracting medium to break into very fine droplets on hitting the hexane phase and to fall slowly through it in this form, collecting at the bottom of the column from which it was automatically drawn off. Approximately 6 liters of the methyl alcohol phase were passed through 200 cc. of the hexane phase. The vitamin was recovered from the methyl alcohol phase by large dilution with water, causing the dissolved hexane to separate and extract the vitamin. About 75 per cent of the vitamin and about 20 per cent of the total substance originally in the hexane layer were extracted by the alcohol phase. This result is contrary to Dam's statement ((1) p. 652) that on partition between methyl alcohol and petroleum ether the vitamin remains in the hydrocarbon solvent.

Later a successful shorter procedure was employed to eliminate the laborious partition step. This consisted in evaporating the hexane solution to dryness under a vacuum and taking up the solid residue with an equal volume of purified absolute methyl alcohol at about 50°. A reddish mass consisting mostly of carotenoid pigments failed to dissolve and was discarded. The methyl alcohol solution was then chilled for 24 hours or more at - 1.1°. A considerable amount of sterols and reddish pigment separated and was removed by centrifuging. This solid material was redissolved in a minimum quantity of hot absolute methyl alcohol and chilled out as before. The two filtrates were combined, concentrated to 100 cc., and chilled again. The additional solid matter chilled out was removed, recrystallized, and then discarded when found not to be potent. By repetition of this procedure, a solution in 50 cc. of methyl alcohol was obtained which remained clear at - 1.1° on standing for several days and which appeared to have all the antihemorrhagic vitamin of the original extract.

The methyl alcohol solution was then fractionated by dilution with water, chilling at - 1.1°, and centrifuging. The addition of

10 per cent of water caused the separation of about 2 cc. of a reddish oil. This oil was dried under a vacuum and dissolved in hexane. The clear filtrate was also dried under a vacuum and dissolved in hexane. The dissolved material in each of these fractions was determined on aliquot portions by evaporation of the solvent under a vacuum at room temperature and weighing.

Results of these assays showed that the reddish oil isolated as described above contained the antihemorrhagic vitamin in great concentration. It was adequate as a source of the antihemorrhagic vitamin at a level as low as 2 mg. of oil per kilo of diet, while the filtrate residue was inadequate at twice this level. The reddish oil was found to contain 1.3 per cent of saponifiable material and 10 per cent of material precipitable by digitonin. After removal of these components, the antihemorrhagic vitamin remained in the oily fraction, but the potency of this fraction was diminished because of the saponification treatment.

A sample of the oils from which the sterols had been removed by digitonin was bleached with activated magnesium oxide, just enough being used to remove the reddish color. The loss of adsorbed material was 34 per cent. The material not adsorbed retained an oily appearance and a light yellow color when free from hexane. When assayed with chicks at a level of 2 mg. per kilo of diet, it provided adequate anti-hemorrhagic activity. The minimum required dosage has not yet been determined.

A known weight of the sterol-free, reddish oil was dissolved in hexane and bleached by the slow addition of a dilute solution of bromine in hexane. Bromine was absorbed rapidly until the color became a light yellow. The solution was then washed several times with dilute sodium carbonate solution and with water. This preparation proved adequate at a level of 3 mg. of original weight per kilo of diet, but failed at lower levels. The principal coloring matter of the reddish oil was probably carotenoid in nature.

The known properties of this vitamin may be summarized as follows: It is highly soluble in polar and non-polar organic solvents, but not appreciably soluble in water or in 50 per cent alcohol-water solution. It is relatively stable to heat and light. A sample heated in a sealed tube under carbon dioxide for 24 hours at 120° retained its potency apparently unimpaired. Irradiation

of a sample in a sealed tube for 24 hours with a 500 watt electric lamp at a distance of 3 or 4 inches did not destroy its potency. The vitamin is alkali-labile as already shown. It is not readily adsorbed by activated magnesium oxide or activated carbon under the conditions stated above.

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# THE IRON AND COPPER CONTENT OF MILK THROUGHOUT THE SEASON, AS RELATED TO ANEMIA DEVELOPMENT IN RATS\*

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From the time it was first demonstrated that rats soon develop nutritional anemia when fed an exclusive whole milk diet and that this can be prevented by the addition of inorganic iron and copper, considerable interest has been manifested in the amounts of these two minerals in milk. The amount of iron in milk, as reported by various investigators (1), varies from 0.27 mg. per liter to 2.4 mg. per liter; the amount of copper reported (1) varies from 0.12 to 0.20 mg. per liter. These wide variations may be due to the chemical methods used, to contamination after the milk was drawn, or to natural fluctuations due to environmental conditions, such as feed, season of the year, etc.

Since we had for some time been engaged in nutritional anemia studies and since variations in the copper and iron content of milk were used by some laboratories to explain conflicting results, it was felt desirable to attempt to determine the iron and copper content of milk throughout the year and to correlate, if possible, any variations that might be found with the rate of anemia development in rats fed on this milk exclusively. At the same time, since the theory had been advanced that a hematopoietic factor not affected by pasteurization was found in the milk of cows properly fed (2), opportunity was taken to study particularly the effect of pasture feeding, which might be considered ideal, on the rate of anemia development in rats fed raw milk from pasture-fed cows and pair mates fed pasteurized milk from the same source.

\* Published with the permission of the Director of the Ohio Agricultural Experiment Station.

## EXPERIMENTAL

Ten cows, seven Jerseys and three Holsteins, were selected towards the end of the winter to furnish the milk for these studies. From March 13 until May 1 all the cows received a regular winter ration consisting of alfalfa hay, corn silage, corn, oats, bran, and linseed oil meal. On May 1 the cows were turned out to pasture. All received the same grain mixture until June 1, when some were placed on a low protein mixture and some on a high protein mixture. This continued until October 24, at which time the cows were made available for other work. From that point on various roughages and grain mixtures were fed. There were thus created in the course of the year the usual variations that occur in practical feeding, plus others of an experimental nature. An aliquot sample was taken from each of the cows at the afternoon and morning milkings when samples were desired. The cows were milked into enameled pails and after the milk was weighed the required sample was transferred to a large glass bottle. Half of the total sample was pasteurized ( $62.5^{\circ}$  for 30 minutes) in a closed glass container, cooled, and stored in the refrigerator with the raw half of the sample until used.

Copper and iron determinations were made on the milk at approximately biweekly intervals. This sample represented 1 day's production of all the cows. The method of Callan and Henderson (3), with a few modifications based chiefly on the suggestions of Williams (4), was used for copper, and that of Stugart (5) for iron. Each sample was run in triplicate, one of each trio serving as a recovery of a known added amount of copper or iron. The average recovery of iron was 97 per cent and of copper 95 per cent. For the sake of brevity the results are shown by periods (Table I), the values for each period being the averages of all the samples run during that time. While the group figures indicate rather small variations throughout the season, it should be pointed out that the range, based on individual determinations, was from 0.305 to 0.442 mg. per liter for iron, and from 0.112 to 0.222 mg. per liter for copper.

Starting on March 13, 1933, and on each of the first dates of each period indicated in Table I, pairs of rats were fed this milk exclusively, one rat of each pair receiving raw milk and one pasteurized milk. The rate of anemia development was followed by

weekly hemoglobin determinations, made with the improved Newcomer Bausch and Lomb hemoglobinometer. The data thus obtained are included and treated in Table I.

Based upon the weekly decrease in hemoglobin, which in turn is based on the length of time for the hemoglobin level to drop to 3.0 gm. per 100 cc., it would seem that there was considerable

TABLE I  
*Iron and Copper Content of Milk As Related to Anemia Development in Rats*

Period	Iron	Copper	Initial Hb		Wks. to reach 3.0 gm. Hb		Weekly decrease in Hb	
			Raw milk	Pasteurized milk	Raw milk	Pasteurized milk	Raw milk	Pasteurized milk
	mg. per l.	mg. per l.	gm. per 100 cc.	gm. per 100 cc.			gm.	gm.
May 1, 1933	0.51*	0.17	(6)† 8.2	7.6	6.5	6.5	0.80	0.71
May 20	0.53*	0.14	(5) 8.8	8.7	4.5	6.0	1.29	0.95
May 2-July 5	0.36	0.15	(5) 9.0	9.4	5.0	6.0	1.20	1.07
May 17-July 12	0.36	0.14	(5) 8.9	8.8	5.0	5.0	1.18	1.16
June 21-Aug. 15	0.37	0.14	(6) 7.5	7.2	5.5	4.5	0.82	0.93
July 20-Sept. 8	0.40	0.15	(6) 7.2	7.2	5.5	6.5	0.75	0.65
Aug. 25-Oct. 21	0.42	0.15	(5) 6.6	6.8	7.0	7.5	0.52	0.51
Oct. 4-Dec. 24	0.42	0.17	(4) 7.3	7.3	6.5	6.5	0.66	0.66
Nov. 7-Jan. 4, 1934	0.43	0.19	(3) 8.9	8.2	5.5	6.0	1.07	0.87
Jan. 3-Mar. 1	0.38	0.16	(8) 6.4	7.2	4.5	4.5	0.76	0.93
Mar. 27	0.34	0.16	(5) 7.5	7.3	5.0	4.0	0.90	1.08

*Ration of Cows*—March 13 to May 1, 1933, alfalfa hay, corn silage, and grain; May 1 to October 24, 1933, pasture and grain; October 24 to March 27, 1933-34, various combinations of roughages and concentrates.

\* These values are probably somewhat higher than actual, as further refinements in technique were made later. During corresponding periods of the following year the average iron content was 0.36 mg. per liter.

† Number of pairs.

variation in the rate of anemia development. However, it has been our experience that it will require from 4 to 6 weeks for the hemoglobin of rats of the same litter to drop to 3.0 gm. per 100 cc. Consequently, the only figures that might possibly be significant are those for the periods August 25 to October 21 and October 4 to December 24. It also happens that the iron content of the milk during these two periods was rather high.



On the other hand, both iron and copper were higher in the period of November 7 to January 4, and yet the rate of anemia development was more rapid.

The biological responses seem to confirm the chemical data; namely, that there was not sufficient variation in the copper and iron content of milk from period to period to affect appreciably the rate of anemia development. These results also demonstrate that no hematopoietic factor destroyed by pasteurization is imparted to milk when cows are under so called ideal feeding conditions.

TABLE II

*Computed Intakes of Iron and Copper on Pasture and on Dry Feeding*

Ration	Fe intake	Cu intake
<i>lbs</i>	<i>gm</i>	<i>gm.</i>
100 Pasture (blue grass)	0 89	0 028
5 Grain	0 13	0 022
	1 02	0 050
100 Pasture (alfalfa)	1 36	0 034
5 Grain	0 13	0 022
	1 49	0 056
10 Alfalfa hay	1 97	0 100
30 Corn silage	1 24	0 013
10 Grain	0 26	0 044
	3 47	0 157

Although it had already been demonstrated that the iron and copper content of cow's milk could not be increased by increasing the intake of these minerals (6-8), the data in Table II, based upon chemical determinations of the materials listed, offer additional evidence of the authenticity of this contention. While the cows used in this work were on pasture, they received an average of 5 pounds of grain daily, and a daily consumption of 100 pounds of pasture (equivalent to 20 pounds of dry matter) was assumed. When on dry feed, a typical average daily ration consisted of 10 pounds of alfalfa hay, 30 pounds of corn silage, and 10 pounds of grain.

It will be seen from Table II that the iron and copper intake when the cows were on dry feed was approximately 3 times that obtaining when they had access to pasture. This wide range in intake was not reflected in the iron and copper content of the milk. The chemical variations found in the milk were, therefore, normal variations which might to a considerable degree be explained on the basis of errors in the chemical procedure.

There was no difference in growth response on either raw or pasteurized milk at any season of the year. Owing to the disturbing influence of anemia development this observation may be of no significance. It was observed, however, that during the dry portion of the pasture season the growth peak was definitely lowered on each kind of milk.

#### SUMMARY

The iron and copper content of pooled milk from Jersey and Holstein cows was determined at approximately biweekly intervals throughout the season. Milk from the same cows was used throughout, except when through dryness, illness, or other reason, milk from one or more individuals was not available. Stugart's method was used for iron and the Callan and Henderson method (modified) for copper.

Slight variations in both iron and copper were found, but these could not be correlated with any change in feeding program.

Groups of rats were started at approximately monthly intervals on milk exclusively, this milk being of the same supply as that on which the iron and copper determinations were made. Nutritional anemia was produced, and at approximately the same rate, on milk produced under any of the various conditions of feeding and management. The biological response of rats and the iron and copper determinations offer evidence of the rather uniform iron and copper content of milk, regardless of different feeding and environmental conditions.

Paired feeding was used throughout, one rat of each pair receiving pasteurized milk. The similar biological response of pair mates indicated that even under pasture-feeding conditions no hematopoietic factor that is destroyed by pasteurization is present in milk.

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## TRYPTOPHANE METABOLISM

### VIII. GROWTH AND KYNURENIC ACID PRODUCTION ON CARBONIC ACID DERIVATIVES OF TRYPTOPHANE\*

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Two criteria are available for estimating the apparent liberation of tryptophane *in vivo* from compounds of tryptophane: (1) growth of young rats receiving a tryptophane-deficient diet supplemented with the tryptophane derivative and (2) kynurenic acid production following the administration of such a derivative. Employing both of these, we have already studied several peptide-like tryptophane derivatives. The data at hand on such compounds are still too sparse to permit any safe generalization as to the influence of chemical structure on their *in vivo* cleavage. We have, therefore, undertaken to extend these studies by testing several series of untried derivatives of different types.

Several peptide-like *l*-tryptophane derivatives previously studied readily replaced tryptophane in the diet of the rat for purposes of growth; these were acetyltryptophane (Berg, Rose, and Marvel, 1929-30; du Vigneaud, Sealock, and Van Etten, 1932; Berg, 1934), propionyltryptophane, phenylpropionyltryptophane (Berg and Hanson, 1934), and substituted and unsubstituted amides (tryptophaneamide, ethylamide, diethylamide, anilide, and ethylanilide (Bauguess and Berg, 1934). On the other hand  $\beta$ -indole- $\alpha$ -uraminopropionic acid (Jackson, 1929), benzoyltryptophane (Berg, Rose, and Marvel, 1929-30), and phenylacetyltryptophane (Berg and Hanson, 1934) were ineffective in promoting growth.

\* A portion of this communication was presented before the American Society of Biological Chemists at New York, March 30, 1934 (*Proc. Am. Soc. Biol. Chem.*, **8**, viii (1934); *J. Biol. Chem.*, **105** (1934)).

The experimental data are taken from a dissertation submitted by Lyle C. Bauguess in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa.

All of the amides were converted to kynurenic acid as efficiently in the rabbit as was *l*-tryptophane, itself. The acetyl derivative yielded considerably less kynurenic acid than did *l*-tryptophane (Berg, 1931, 1934), and the benzoyl derivative none. The phenylacetyl, propionyl, and phenylpropionyl derivatives and indoleuraminopropionic acid have not yet been similarly tested.

In the present study several substituted carbonic acid derivatives of *l*-tryptophane were chosen, which were closely related to the acyl derivatives previously employed; these were the methyl, ethyl, propyl, phenyl, and benzyl carbonates. Such compounds differ from the derivatives previously studied only in that the alkyl or aryl group is linked through an atom of oxygen to the carbonyl group, instead of being directly connected. All were tested from the standpoints both of growth and of kynurenic acid production.

#### EXPERIMENTAL

The carbonic acid derivatives were prepared by the interaction of an alkaline solution of *l*-tryptophane with the appropriate chlorocarbonate.

The methyl, ethyl, and propyl chlorocarbonates were obtained from the Eastman Kodak Company. The benzyl chlorocarbonate was prepared from phosgene and benzyl alcohol as directed by Bergmann and Zervas (1932). The average yield of three runs was 59.7 gm. (84.1 per cent of the theoretical). The phenyl chlorocarbonate was synthesized essentially according to the procedure outlined by Raiford and Inman (1934). The crude product was distilled *in vacuo*. The fraction boiling at 88–90° (22 mm.) was reserved. It weighed 33.0 gm., representing a yield of 60.6 per cent of the theoretical. Hocflake (1921) records the boiling point as 83–84° at 12 mm.; Inman (1933), 88–90° at 22 mm.

In the syntheses of the tryptophane derivatives the general procedure of Bergmann and Zervas (1932) for preparing carbobenzoxy derivatives of amino acids was followed. The method is essentially a modification of the Schotten-Baumann technique. The crude derivatives were washed several times with 5 to 10 cc. portions of cold water, suspended in 30 cc. of *N* hydrochloric acid solution to dissolve any unchanged tryptophane which may have

been present, again filtered, washed free of chlorides, and dried first at 60° and finally at room temperature over sulfuric acid. Average yields of the several compounds varied from 78 to 89 per cent of the theoretical. The carbomethoxy, carbopropoxy, and carbobenzoxy derivatives can be purified by dissolving in alcohol, cooling well, and adding cold water to effect precipitation. The carboethoxy- and the carbophenoxytryptophane frequently precipitate as gums when their alkaline solutions are acidified. Purification of these two is difficult, but can be accomplished by dissolving them in ether, adding several volumes of benzene, and evaporating off the ether *in vacuo*. As the ratio between the ether and benzene concentrations decreases, the compound gradually precipitates.

TABLE I

*Melting Points and Nitrogen Analyses of Tryptophane and Tryptophane Compounds*

Compound	Melting point (uncorrected)	Nitrogen	
		Found	Calculated
	°C.	per cent	per cent
Tryptophane. . . . .	278	13.70	13.72
Carbomethoxytryptophane. . .	149-150	10.80	10.68
Carboethoxytryptophane. . . .	105-106	10.04	10.14
Carbopropoxytryptophane. . .	124-125	9.68	9.65
Carbophenoxytryptophane. . . .	Decomposed at 151	8.60	8.62
Carbobenzoxytryptophane. . . . .	122-124	8.35	8.26

Melting points and nitrogen analyses of the tryptophane and the compounds appear in Table I.

*Growth Studies*—In testing the compounds for growth-promoting ability, they were employed as supplements in a tryptophane-deficient diet in amount equivalent to 0.2 per cent of tryptophane. Of five litter mates used in testing each compound two served as controls, one receiving no basal diet supplement, the other 0.2 per cent of *l*-tryptophane. Three rats were fed the derivative. The basal diet was composed of acid-hydrolyzed casein 14.7, cystine 0.3, starch 39.5, sucrose 15, Crisco 19, cod liver oil 5, salt mixture (Hawk and Oser, 1931) 4.5, and agar 2 per cent, and was fed *ad libitum*. All supplements replaced an equal amount of hydrolyzed casein. Vitamin B complex was furnished daily to each rat in the

form of pills fed separately and containing 200 mg. of yeast<sup>1</sup> and 100 mg. of starch. Water was always available.

Without exception all of the animals on the derivatives lost weight (6 to 14 gm.) during the 80 day feeding period as rapidly as the controls (8 to 12 gm.) which received no tryptophane. The tryptophane-fed rats gained 99 to 116 gm. in the same period. Food consumption data obtained show a daily intake of 6.1 to 6.6 gm. for the rats receiving tryptophane as a supplement, 2.4 to 3.0 gm. for the controls fed the unsupplemented diet, and 2.2 to 3.0 gm. for the animals supplied with the derivatives as supplements in the food mixture.

*Kynurenic Acid Studies*—Each of the carbonic acid derivatives was tested for kynurenic acid production by administration as the sodium salt, either orally or subcutaneously, to each of four male rabbits in amounts molecularly equivalent to 1 gm. of tryptophane. The 24 hour urines following were collected and analyzed for kynurenic acid according to the Capaldi (1897) procedure. The coprecipitated derivatives were removed by washing with 5 cc. of water-saturated *n*-butyl alcohol. Details of the experimental technique may be found elsewhere (Berg, 1931). The compounds were administered every 3rd day. All 24 hour urines, whether collected on experimental or on intervening days, were analyzed routinely.

No appreciable amount of kynurenic acid was obtained after the administration of any of the five derivatives in any of the four separate tests made of each. The extreme variations in apparent kynurenic acid output after the administration of the several compounds ranged from 0.0012 to 0.0095 gm., as compared with variations from 0.0023 to 0.0080 gm. during the intervening periods and from 0.2862 to 0.3571 gm. after 1 gm. of tryptophane was given. The average weight of apparent kynurenic acid in the twenty trials on the compounds was 0.0045 gm., as compared with an average of 0.0046 gm. in the 52 tests on intervening periods when no administrations were made and an average of 0.3157 gm. in the four periods after tryptophane was given. On the other hand, appreciable quantities of each of the derivatives, varying from 21 to 114 mg., were obtained by evaporating the butyl alcohol washings of the

<sup>1</sup> The yeast was kindly supplied by the Northwestern Yeast Company, Chicago.

Capaldi precipitate to dryness. In all cases the recovered product, alone or mixed with a sample of the original carbonic acid derivative, melted at a temperature very close to the melting point of the original product.

Apparently none of the compounds underwent cleavage in either the rat or the rabbit under the experimental conditions employed. This is in striking contrast to the observations previously cited that the corresponding acetyl and propionyl derivatives of tryptophane are utilizable for growth and that the acetyl derivative yields kynurenic acid.

#### SUMMARY

Several carbonic acid derivatives of tryptophane (carbomethoxy-, carboethoxy-, carbopropoxy-, carbophenoxy-, and carbo-benzoxy-N-tryptophane) have been prepared. Each has been tested for growth-promoting ability in the young rat and for kynurenic acid production in the rabbit. None was effective in either capacity. Apparently none of the compounds can be hydrolyzed in these animals.

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## THE CALCIUM AND PHOSPHORUS CONTENT OF THE BODY OF THE BROOK TROUT IN RELATION TO AGE, GROWTH, AND FOOD

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For nearly 100 years it has been the practice of trout hatcheries to feed diets rich in meat. Brook trout can be reared to maturity upon a diet of beef liver, which is rich in phosphorus and poor in calcium. No one has reported any pathological condition from such diets. No disease similar to rickets is known to afflict trout.

In an earlier study (1) of the chemical composition of the bodies of brook trout it was found that the calcium and phosphorus of the body varied in relation to the diet but within rather narrow limits. Trout fed diets rich in phosphorus and poor in calcium for periods of many months grew normally, and in the end the level of these inorganic constituents of their bodies reached about the same level as those fed diets supplemented with calcium.

At a moderate rate of growth a trout weighing 30 gm. will increase its body weight by 10 to 20 gm. in the course of 10 weeks. The body of a trout of this size contains 0.42 to 0.58 per cent of calcium. Then an increase of 10 gm. in body weight is accompanied by a storage of 0.04 gm. of calcium. Assuming more inefficient conversion than is usually observed, an increase of this amount is made when 80 gm. of beef liver are fed. This amount of liver contains about 0.009 gm. of calcium. Therefore, the water must provide 0.031 gm. of calcium during this period.

To test these calculations three groups of trout, containing 50 individuals in each, were placed in isolated troughs. All were fed a diet of beef liver. At the end of 5 days twenty-five trout from each group were preserved for analysis. The remaining

trout were fed the same liver diet for the next 12 weeks. They were then killed for analysis. Samples of water were taken each day during this period and preserved in paraffined bottles. At the conclusion of this experiment the trout, the liver, and the water were analyzed for calcium by the method of Morris, Nelson, and Palmer (2).

The data from this experiment are summarized in Table I. The growth of the whole body during this trial was subnormal. However, the bones continued to grow. Calcium was stored in liberal amounts. About three-fourths of this calcium came from the water rather than the food. The water during this period was quite constant at 43 mg. of Ca per liter.

TABLE I  
*Relative Amounts of Ca, from Water and Feed, Stored during Growth  
in Body of Brook Trout*

Group No	Mean weight at beginning	Mean gain in weight of live trout per 12 wks.	Ca in 25 fish at start	Ca in 25 fish at end	Difference equals Ca stored in 3 mos	Ca stored per trout	Raw liver fed for 3 mos	Ca fed in liver	Ca taken from water containing 43 mg. per liter
	gm.	gm.	mg.	mg	mg.	mg.	gm.	mg.	mg.
56	3 8	0 5	360 3	495 5	135 2	5 4	478	27 9	107.3
57	3 6	0 8	337.1	491 3	154 2	6 2	434	25 3	128 9
58	3 7	0 9	368 6	516 7	148 1	5 9	488	28.5	119 6

The water used in these experiments is taken directly from a large spring originating in a hill of gravel. This water supply remains at a constant temperature of 9° throughout the year, with a variation of less than 0.5°.

To learn more concerning the changes that occur in the phosphorus and calcium during the early stages of the development of fish, a second series of experiments was devised 2 years after the completion of those described above. The purpose of these experiments was to determine the changes that take place in the body of the trout from the time the egg is produced until the fish has hatched and developed into its final form.

Eggs were taken from special breeders and segregated in regular hatching troughs in water at a temperature of 9°. 1 day after the eggs were taken 500 of them were set aside, preserved in

TABLE II  
*Changes with Age in Calcium and Phosphorus in Bodies of Brook Trout*

Date of period	Material used	Mean fresh weight at start of period	Mean fresh weight at end of period	Food fed per specimen during period	No of specimens pooled for analysis	Ca per specimen at start	Ca per specimen at end	Ca gained by body for period	Ca ingested in food for period	Ca taken from water in period	P per specimen at end
1883											
Nov. 27	Eggs	0.024			500	0.008				mg.	mg.
1884											
Jan. 15	Eyed eggs				500	0.010					0.079
" 30	Sac fry	0.03			500	0.014					0.088
Mar. 9	Fry	0.05			500	0.035					0.100
" 9-Apr. 20	Fingerling	0.05	0.08	0.26	500-50	0.035	0.097	0.062	0.025	0.037	0.106
Apr. 20-June 15	"	0.08	0.45	0.82	50-50	0.097	0.927	0.830	0.078	0.752	1.040
June 15-July 13	"	0.45	0.65	0.96	50-50	0.927	1.852	0.925	0.090	0.835	2.17
July 13-Aug. 10	"	0.65	1.27	1.18	50-20	1.852	3.429	1.577	0.111	1.466	3.68
Aug. 10-Sept. 7	"	1.27	2.20	2.13	20-15	3.429	7.177	3.748	0.201	3.547	6.80
Sept. 7-Oct. 5	"	2.20	3.05	4.15	15-5	7.177	8.832	1.655	0.391	1.264	9.30
Oct. 5- " 16	"	3.05	3.78	1.84	5-15	8.832	10.790	1.958	0.173	1.785	11.70
" 16-Nov. 2	"	3.78	5.50	3.06	15-15	10.790	18.542	7.752	0.288	7.464	20.40

alcohol, for analysis. At a later date, when the "eyed" stage was reached, another sample was preserved. 2 weeks later, when these eggs had hatched, a sample of the "sac fry" was taken. Another sample of these fry was preserved just as the egg sac was absorbed and just before the young trout were ready to eat. After this, samples were taken at intervals of 4 weeks. This experiment was run in triplicate but the results were very similar and only one set of data are included. Ground beef liver was used as the sole diet. A large supply of this liver was mixed with 10 per cent of its weight of alcohol and kept in cold storage for the 8 months that it was used for feed. This liver contained 9.4 mg. of calcium and 310 mg. of phosphorus per 100 gm. of fresh material.

Analyses were run by the same method of Morris, Nelson, and Palmer (2). The water was analyzed from composites taken each day and preserved in paraffined bottles. The water supply proved to be very constant in its content of calcium. This varied between 44 and 46 mg. per liter. The amount of food eaten by the trout was recorded each day.

All pertinent data are summarized in Table II. These indicate that much more calcium is taken from the water than is provided by the food supply. Before the young trout starts to eat the phosphorus of the body is several times the calcium in amount. Even before the young fry is able to eat, however, and while it is still building its body from the yolk sac, it is taking calcium from the water in which it swims. Shortly after the young fry starts to eat it absorbs calcium very rapidly until the calcium of its body reaches a level nearly equal to the phosphorus. After the young trout begins to eat the increase of these elements is more rapid than the growth of the entire body. These data are similar in many respects to those found by Sherman and Quinn in the case of the rat (3).

#### SUMMARY

In a preliminary experiment brook trout were analyzed for calcium at the beginning and end of a 10 week period. Only about one-fourth of the calcium during this period originated in the food and the remainder must have been absorbed from the water. In a second series of experiments calcium and phosphorus

were determined, starting with eggs 1 day old and ending with trout 10 months old. Even during the stage when the egg sac is being absorbed and before the young fish has started to eat, there is a marked increase in the body calcium which must originate in the water. In the eggs and fry there is much more phosphorus than calcium, but as soon as the fry starts to feed, there is a rapid increase in this element until it is nearly equal to the phosphorus of the body.

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## OXIDATION OF AMINO ACIDS BY *BACILLUS PYOCYANEUS* (*PSEUDOMONAS AERUGINOSA*)

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A few studies have been made of the intermediate products of the amino acids oxidized by *Bacillus pyocyaneus*. Raistrick and Clark (1) showed that the indole ring of tryptophane was broken and that tyrosine was attacked. Their work was continued by Supniewski (2). Arginine was transformed to citrulline by a specific enzyme by Horn (3). We have studied the oxidation of various amino acids by *Bacillus pyocyaneus* by quantitative measurements of the oxygen uptake, carbon dioxide production, deamination, and the effects of various drugs. For our experiments a washed suspension of the bacteria was used, but in contrast to *Bacillus proteus* (4) washing did not produce a completely resting state.

No evidence of growth was obtained during the experiment. Moreover, the length of the experiment (2 to 4 hours) and the unfavorable conditions for growth during it exclude this possibility. The oxygen uptakes ended abruptly when the theoretical amounts of oxygen had been utilized. Unlike *Bacillus proteus*, *Bacillus pyocyaneus* showed a haphazard optical specificity, differentiating sharply between the optical isomers of certain amino acids but oxidizing both isomers of others.

### EXPERIMENTAL

*Bacillus pyocyaneus* was grown for 18 hours at 37° on beef extract agar. The bacteria were washed off with saline and cen-

\* The work reported in this paper was done in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology and Pharmacology, Duke University and Medical School.



trifuged four times in Hopkins tubes. They were then suspended in 0.05 M phosphate buffer, pH 7.8, so that there were approximately 7 billion per cc. 0.5 cc. of this suspension was used in each Warburg vessel and the volume made up to 2 cc. with a buffered solution of the amino acid. The oxygen uptake and the CO<sub>2</sub> production were determined in the usual way. The ammonia production was measured at the end of the experiment by vacuum distillation followed by Nesslerization.

*O<sub>2</sub> Uptake and CO<sub>2</sub> Production*—Table I summarizes the results. The amino acids utilized different amounts of oxygen. For instance there was a marked contrast between tyrosine and histidine, for the former utilized 11 atoms of oxygen per molecule and the latter only 4. Isoleucine is oxidized further than leucine and valine further than serine. These facts suggest either that the bacteria normally used the residues for other purposes and thus do not oxidize them to CO<sub>2</sub> and H<sub>2</sub>O or that in the process of washing the catalysts responsible for further oxidation are destroyed. In our experiments the CO<sub>2</sub> production paralleled the oxygen uptake, so that the process involved was the breakdown of larger molecules to smaller ones and was not the interconversion by oxidation of one type of molecule into another with the same number of carbon atoms. Fig. 1 shows that the oxygen uptakes stop off sharply.

*Optical Specificity*—The optical specificity of *Bacillus proteus* was sharply defined (4). Both isomers of alanine and serine were oxidized and there was an indication that the non-natural isomer of valine, though not oxidized, could combine with the catalyst and inhibit the oxidation of the natural isomer. But as the molecule became larger by the introduction of another methyl group as in leucine, definite optical specificity occurred and only the natural isomers could be oxidized. This is not true of *Bacillus pyocyaneus*. With these bacteria the amino acids could be divided into three classes. The first class consisted of leucine, isoleucine, and histidine, which show definite optical specificity and only the natural isomers are attacked. The second class consisted of phenylalanine and valine. With these two amino acids the non-natural isomers definitely increased the oxygen uptake of the bacteria and this increase was proportional within limits to the concentration of the

TABLE I

*Oxidation, Decarboxylation, and Deamination of Various Amino Acids at 37°, pH 7.8*

The oxygen uptake and decarboxylation as shown in Columns 2 and 6 are calculated for both isomers. For determining the amount of deamination 1.0 mg. instead of 0.25 mg. was used in each case and the theoretical deamination shown in Column 9 is calculated on that basis. The oxidation rate (Column 4) is based on the time necessary to reach half the final uptake.

Amino acid (0.25 mg. used)	Observed O <sub>2</sub> uptake	Calculated O <sub>2</sub> uptake	O <sub>2</sub> utilized	Oxidation rate	Observed CO <sub>2</sub>	Calculated CO <sub>2</sub>	CO <sub>2</sub>	Observed NH <sub>3</sub> -N	Calculated NH <sub>3</sub> -N
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	c mm.	c.mm.	atoms	min.	c.mm.	c mm.	mole- cules	mg.	mg.
<i>dl</i> -Leucine . . . . .	62	126	3	110	53	92	2	0.066	0.053*
<i>d</i> -Leucine . . . . .	9	0	0	0	0	0	0	0.000	0.000
<i>l</i> -Leucine . . . . .	125	126	6	133	75	92	2	0.088	0.107
<i>dl</i> -Isoleucine . . . . .	96	168	4	54	63	138	3	0.067	0.053*
<i>d</i> -Isoleucine . . . . .	171	168	8	66	126	138	3	0.100	0.107
<i>l</i> -Isoleucine . . . . .	15	0	0	0	0	0	0	0.000	0.000
<i>d</i> -Histidine . . . . .	3	0	0	0	0	0	0	0.000	0.000
<i>l</i> -Histidine . . . . .	69	72	4	170	72	73	2	0.150	0.088†
<i>dl</i> -Valine . . . . .	151	144	6	85	72	150	3	0.070	0.060*
<i>d</i> -Valine . . . . .	174	168	7	91	144	150	3	0.097	0.120
<i>l</i> -Valine . . . . .	45	48	2	80	6	0	0	0.000	0.000
<i>dl</i> -Phenylala- nine . . . . .	176	170	10	98	92	140	2	0.063	0.042*
<i>d</i> -Phenylalanine . . . . .	77	68	4	120	110	105	3	0.000	0.000
<i>l</i> -Phenylalanine . . . . .	223	221	13	127	132	140	4	0.082	0.085
<i>dl</i> -Alanine . . . . .	125	126	4	19	114	128	2	0.148	0.079*
<i>d</i> -Alanine . . . . .	122	126	4	19	112	128	2	0.167	0.158
<i>dl</i> -Serine . . . . .	86	41	3	33	48	57	1	0.127	0.066*
<i>dl</i> -Proline . . . . .	126	168	5	104	92	98	2	0.113	0.060*
<i>l</i> -Proline . . . . .	169	168	7	105	119	98	2	0.093	0.121
<i>dl</i> -Tyrosine . . . . .	157	170	11	102	162	155	5	0.077	0.038*
<i>l</i> -Tyrosine . . . . .	169	170	11	78	171	155	5	0.073	0.077
<i>l</i> -Oxyproline . . . . .	101	105	5	182	80	76	2	0.082	0.107
Glycine . . . . .	113	112	3	57	82	70	1	0.160	0.186

\* Theoretical for one isomer.

† Theoretical for 1 nitrogen atom per molecule.

isomer used. No deamination, however, occurred. There is therefore a possibility that the non-natural isomer is not directly attacked, but that its presence stimulates the residual oxygen uptake of the bacteria. There is some evidence from the oxygen uptake figures that the non-natural isomers of these two amino acids may be somewhat more readily oxidized when present in

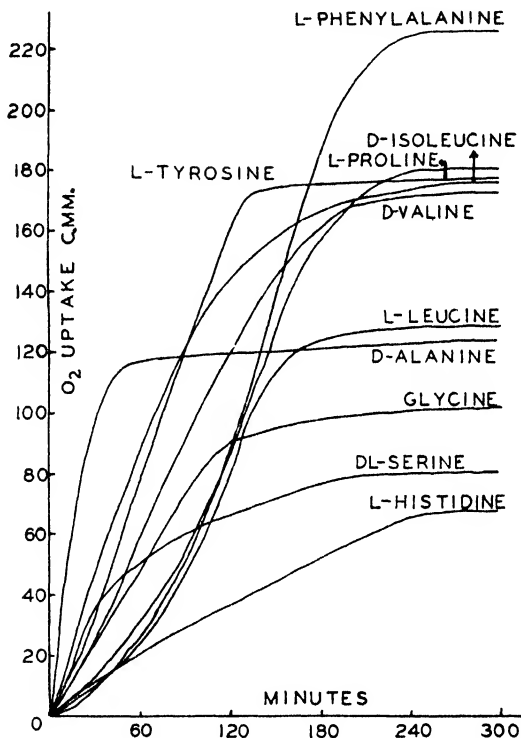


FIG. 1. Oxidation rate of various amino acids by *Bacillus pyocyaneus*

the *dl* mixtures, but under these conditions also no deamination takes place (see Table I). The conclusion is that these two isomers have a definite effect on the oxygen uptake of the bacteria, but whether they undergo oxidation without deamination or act as a stimulus to the residual respiration of the bacteria is difficult to decide.

The third class consisted of alanine, serine, tyrosine, and pro-

line. Although the non-natural isomers of these amino acids were not available, the figures obtained from the oxygen uptake and the ammonia production of the natural and *dl* mixtures show that both isomers were readily attacked. Thus if only one isomer of *dl*-proline were attacked, 0.60 mg. of ammonia would have been produced. Actually 0.113 mg. was found. Similar figures were found for alanine and tyrosine. Only *dl*-serine was used, but here also the ammonia production showed that both isomers were deaminated. The oxygen uptake figures for the *dl* mixtures of tyrosine and proline showed that the non-natural isomers are oxidized to a somewhat less extent, even though complete deamination of both takes place. In the case of alanine, however, both isomers were equally well oxidized. Inasmuch as there is no structural similarity between proline, tyrosine, serine, and alanine which distinguishes them from the other amino acids, the incidence of optical specificity and non-specificity in this class of compounds must at present be considered haphazard.

*Action of Drugs and Dyes*—KCN in concentrations of 0.005 M completely inhibited the oxidation of all of the amino acids. This was also true for *Bacillus proteus*. The action of KCN on the oxidation of amino acids by mammalian organs is sharply differentiated. The natural isomers are cyanide-sensitive (5, 6). No such differentiation occurred in the two species of bacteria studied. Evidently the oxidation mechanism for both isomers in these bacteria is the same and is dependent upon iron in some form.

Sodium fluoride in concentrations up to 2 per cent and urethane in concentrations up to 5 per cent were tried in order to see whether the residual respiration of the bacteria could be inhibited without affecting the oxidation of the amino acids. It was found, however, that the residual respiration and the amino acid oxidation fell off proportionately as the concentrations of the drugs were increased. With intermediate concentrations, great enough markedly to inhibit mammalian tissue, no inhibition of the amino acid oxidation occurred. In a few cases accelerations were observed.

The effect of the amino acids on the reduction time of methylene blue was complicated by the fact that the bacteria themselves reduced the dye. So it was impossible to determine the relative reduction rates with the different amino acids, as in the case of *Bacillus proteus* (4). The addition of the amino acids to the

*Bacillus pyocyaneus* decreased the reduction time slightly in all cases but no one amino acid was more markedly reducing than another. Variations in the amino acid, bacteria, and methylene blue concentrations had no effect. Therefore dyes of other potentials were tried, such as the indigo sulfonates, in an attempt to bring out differences in the reduction rates if they existed. Although the absolute rates were, of course, different with the different dyes, the relative reduction rates remained the same. The conclusion from these facts is that all the natural and non-natural isomers that are oxidized can utilize certain dyes as well as oxygen as hydrogen acceptors, but that when added to the bacteria they change their potential so slightly that marked differences in reduction rates are not seen.

Because pyocyanine is one of the pigments produced by *Bacillus pyocyaneus* and since it is reversibly oxidized and reduced, it might possibly affect the oxidation of the amino acids. In concentrations of 1:10,000 it had, however, no effect on the residual respiration of the bacteria or on the rate of oxidation of several amino acids. Nor did it cause the oxidation of either tryptophane or methionine, the two amino acids which were not attacked by our strain of *Bacillus pyocyaneus*. Two other strains isolated from clinical cases of infection also failed to oxidize these amino acids.

#### DISCUSSION

There are three general methods of studying the metabolism of bacteria. The first is by measuring the metabolism under normal conditions of growth and reproduction. This has been done in a large number of cases and very successfully by Martin (7). The second is the inclusion of the specific substance to be studied in the medium and the isolation of the products after a period of incubation. This method may yield by-products rather than intermediate products. The third is the study of the metabolism of specific substances under conditions where other metabolic activities of the bacteria are suppressed or reduced to a minimum. The last method eliminates the complicating factor of growth. The preceding study, which was carried out according to the third method, has shown that each amino acid is attacked in a different way. In the first place the rate of oxidation of the various amino acids varies markedly. This may mean that there are separate

catalysts for each amino acid and that these are affected differently by the treatment of the bacteria, or more probably that the rate of formation of the enzyme-substrate complex is determined by the chemical structure of the amino acid. In the second place, the optical specificity varies in different groups of amino acids and the specificity of *Bacillus pyocyaneus* is quite different from that of *Bacillus proteus* under similar conditions. Why such differences should exist is difficult to explain, and a large number of studies on the optical specificity of different bacteria are needed before any general rules can be applied. It is evident, however, that bacteria which attack amino acids do not show strict optical specificity.

#### SUMMARY

1. The oxidation of thirteen amino acids by washed *Bacillus pyocyaneus* was studied. Of these amino acids tryptophane and methionine were not attacked.

2. There are large variations in the oxidation rates and the extent to which the different amino acids are oxidized.

3. The bacteria oxidize and deaminate only the natural isomers of leucine, isoleucine, and histidine but both isomers of alanine, serine, tyrosine, and proline. The non-natural isomers of valine and phenylalanine are slightly attacked.

4. The oxidation and deamination of both isomers are completely inhibited by 0.005 M KCN, but are unaffected by 1 per cent sodium fluoride or 1 per cent urethane.

5. Methylene blue is reduced by all the amino acids but no differences in relative rates occurred. Neither this dye nor pyocyanine added to the bacteria affected the oxidation.

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# THE ACCELERANT EFFECT OF $\alpha$ -AMINO ACIDS ON THE ACTIVITY OF BONE PHOSPHATASE

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$\alpha$ -Amino acids in *very low* concentrations have been reported to exert an accelerant effect on the activity of urease (1-7), the several amylases (8-14), and pancreatic lipase (15). Interest attaches, of course, to the mode of such acceleration. But since  $\alpha$ -amino acids are present as products of autolysis in enzymic extracts of tissues (16, 17), there also arises the problem of evaluating the influence of these acids on the estimation of the enzymic activities of such extracts.

In the present study it is shown that  $\alpha$ -amino acids increase the activity of bone phosphatase. The mechanism of the acceleration is then studied and the conditions determined under which the velocity of the enzymic reaction is a precise measure of the concentration of bone phosphatase present.

## EXPERIMENTAL

The phosphatase preparations were obtained by a 2 to 3 day autolysis at room temperature. The leg bones (usually the tibia and fibula) of the young animal were stripped of adherent tissue, and broken or ground into small fragments. For the extraction of the cattle or lamb bones, 0.5 cc. of toluene and 10 cc. of distilled water were added for each gm. of bone; for the preparation of the rat bone phosphatase, 20 cc. of distilled water and 1 cc. of toluene were used per gm. of tissue. The mixtures were occasionally shaken and finally filtered and stored in the ice box; the preparations maintained their activities with only slight decreases for months. Except for the use of toluene, this is essentially the method employed by Kay (18). For dialysis artificial sausage



casings (Visking) were used. The amino acids were either Eastman Kodak Company or Kahlbaum products; solutions of these were made directly, or with the aid of acid or alkali. Magnesium chloride (Baker and Adamson) was the source of magnesium ion.

The hydrolyses were carried out as previously described (19). The substrate was sodium  $\beta$ -glycerophosphate (Eastman); the buffer, sodium diethylbarbiturate. The temperature of the reaction was maintained at  $25^{\circ} \pm 0.05^{\circ}$ ; a few reactions were carried out at room temperature ( $22$ – $25^{\circ}$ ). In the course of this paper, the concentration of enzyme extract is expressed as volumes per cent of the hydrolysis solution. Values for extent of hydrolysis are expressed as mg. of phosphorus liberated as inorganic phosphate per cc. of hydrolysis mixture. Complete hydrolysis yielded 0.394 mg. of phosphorus per cc. To avoid a classic error (20) in the comparison of the activities of varying concentrations of an enzyme extract, it was decided to insure the determination at optimal pH by running a series of four hydrolyses, as previously described (19), in the neighborhood of pH 9.0.

#### *Effect of $\alpha$ -Amino Acids on the Course of Phosphatase Hydrolysis*

The early portion of the course of hydrolysis of glycerophosphate by a 12.5 per cent by volume concentration of cattle bone phosphatase, Preparation CBF, is shown in Table I. The average rate of hydrolysis,  $\Delta p/\Delta t$ , decreases quite rapidly when no  $\alpha$ -amino acid is present. Thus the average rate during the first 10 minutes was 0.00027 mg. per cc. per minute; it decreased to 0.00009 mg. per cc. per minute during the period of 80 to 120 minutes. The presence of 0.00625 M glycine did not change the velocity with which the reaction started but did prevent the marked decrease. The average rate during the period, 80 to 120 minutes, was 0.00017 mg. per cc. per minute. The presence of 0.009 M magnesium ion increased the rate at which the reaction started and also prevented the marked decrease in the subsequent rates.

In measuring the concentration of an enzyme in terms of its reaction velocity, three methods are available: (1) the measurement of initial velocities; (2) the calculation of a reaction constant, usually monomolecular; (3) the calculation, from the course of the reaction, of the reciprocal of the time necessary to effect a given change in a given concentration of substrate. The use of

initial velocities is feasible when the first portion of the reaction is a straight line, as is the case with invertase during the first 10 per cent of the hydrolysis of sucrose (21). In bone phosphatase, as shown above, the hydrolysis of only the first 1 per cent of the glycerophosphate seems to proceed at a constant rate; therefore

TABLE I

*Effect of Glycine and of Magnesium on the Course of Hydrolysis of Sodium  $\beta$ -Glycerophosphate by Bone Phosphatase*

Temperature, 25°; cattle bone phosphatase, Preparation CBF, 12.5 per cent concentration in hydrolysis mixture. The value for the average rate of liberation during a given time period is tabulated in the interspace between the times beginning and ending that period.

Time	P liberated as phosphate per cc. of hydrolysis mixture in presence of			Average rate of liberation of P per cc. per min. in presence of		
	No addition	0.00625 M glycine	0.009 M magnesium	No addition	0.00625 M glycine	0.009 M magnesium
min	mg	mg	mg.	mg.	mg.	mg.
0						
10	0 0027	0 0027	0 0032	0 00027	0 00027	0 00032
20	0 0051	0 0052	0 0063	0 00024	0 00025	0 00031
40	0 0090	0 0095	0 0121	0 00020	0 00022	0 00030
80	0 0134	0 0163	0 0240	0 00011	0 00017	0 00030
120	0 0176	0 0230	0 0343	0 00009	0 00017	0 00026
160	0 0216	0 0294	0 0440	0 00010	0 00016	0 00025
220	0 0273	0 0380	0 0580	0 00009	0 00014	
280	0 0318	0 0463				
340	0 0357	0 0542				

determinations are subject to large error. The hydrolysis by bone phosphatase, as is the case with practically all enzymic reactions, cannot be described as monomolecular. The most widely used measure for the determination of enzymic concentration in terms of activity has been the reciprocal of the time necessary to effect a given change (22, 23). In the use of this measure, as the enzyme

concentration is varied, the concentration of substrate and reaction products, and the effect of the latter, are kept constant.

Following Northrop (23) we have designated the reciprocal of the time as  $Q$ . Unless otherwise indicated,  $Q$  designates in this study  $Q_{0.05}$  or the reciprocal of the time in minutes necessary for the liberation of 0.05 mg. of phosphorus as phosphate per cc. of hydrolysis mixture.

The prevention by glycine of the rapid decrease in the rate of hydrolysis of glycerophosphate by cattle bone phosphatase is reflected in the reciprocal of the time in minutes necessary for the liberation of 0.05 mg. of phosphorus per cc. It was found that

TABLE II

*Dependence of Reaction Velocity on Concentration of Glycine*

Dialyzed cattle bone phosphatase, Preparation CBH-1-d; temperature, 25°.

Concentration of added glycine	$Q_{0.05}$ = reciprocal of time in min. necessary to liberate 0.05 mg. of P as phosphate per cc. of hydrolysis mixture at		$\frac{Q_{0.05} \text{ at 75 per cent}}{Q_{0.05} \text{ at 12.5 per cent}}$
	12.5 per cent phosphatase	75 per cent phosphatase	
<i>M per l.</i>			
0 0	0.0017	0 0235	13.8
0.00031	0 0025	0 0278	11.3
0.00094	0 0035	0 0325	9.3
0.0025	0 0048	0.0400	8.3
0.00625	0.0047	0.0420	9.0
0.025	0 0043	0.0375	8.7
0 050	0 0035	0.0287	8.2

the extent of this prevention depended upon the concentration of glycine (Table II), the maximal effect occurring at a concentration of 0.0025 M to 0.00625 M. At higher concentrations, a retardant effect set in, and  $Q$  decreased. From the data of Table II alone, it is impossible to decide which value of  $Q$  should be taken as representative of the activity of the given concentration of enzyme.

This accelerating effect was found to hold for other  $\alpha$ -amino acids and their derivatives, but not for other nitrogen compounds. Preliminary experiments at room temperature on a weak cattle bone phosphatase, Preparation CBG, showed that cysteine, cys-

tine, glycine, *d*-isoleucine, hippuric acid, glutamic acid, glutathione, histidine, and tyrosine, produced marked acceleration. Without any addition, the time required for the liberation of 0.05 mg. of phosphorus per cc. was about 62 hours. In the presence of an optimal concentration of  $\alpha$ -amino acid, the time required for the liberation of the same amount was about 21 hours. The optimal concentration varied with the  $\alpha$ -amino acid; for the group as a whole it ranged from about 0.01 M to 0.0001 M. Glucose, lactic acid, creatine, creatinine, urea, and *o*-aminobenzoic acid did not accelerate hydrolysis.

In Table III are shown the accelerant effects of several  $\alpha$ -amino acids on a more active cattle bone phosphatase, Preparation

TABLE III  
*Accelerant Effect of Added  $\alpha$ -Amino Acid on Activity of Cattle Bone Phosphatase*

Temperature, 25°; Preparation CBH-2, 12.5 per cent concentration in hydrolysis mixture.

<i>d</i> -Isoleucine		<i>l</i> -Histidine		Glutamic Acid		Glycine	
Concentration	$Q_{0.05}$	Concentration	$Q_{0.05}$	Concentration	$Q_{0.05}$	Concentration	$Q_{0.05}$
<i>M per l</i>		<i>M per l</i>		<i>M per l</i>		<i>M per l</i>	
0.0	0.0047	0.0	0.0047	0.0	0.0047	0.0	0.0047
0.0025	0.0063	0.00028	<b>0.0069</b>	0.00063	<b>0.0065</b>	0.00063	0.0063
0.010	<b>0.0065</b>	0.00075	0.0066	0.0025	0.0064	0.0025	0.0066
0.040	0.0027	0.0030	0.0052	0.0125	0.0060	0.0156	<b>0.0066</b>
		0.0075	0.0017	0.0500	0.0057	0.0625	0.0047

CBH-2. The form of the reaction velocity-log amino acid concentration curve (when it was so plotted) differed for the different amino acids. The zone of optimal activity also varied, but the optimal activity attained was, within experimental error, the same for the different compounds,  $Q_{0.05} = 0.0066$ . Greater concentrations than optimal decreased the activity. Concentrations ranging from 0.1 to 0.01 M, depending upon the particular amino acid, depressed the activity below that of the preparation without any added amino acid. Such an effect was previously reported (19).

The finding that  $\alpha$ -amino acids accelerate the activity of cattle bone phosphatase whereas other compounds containing amino groups do not is, in general, in agreement with the results of Rock-

wood and Husa (6) on urease and Sherman and Naylor (12) on amylase. The former authors studied the effect at only one concentration, 0.001 M, but on a great many compounds. They found that the ammonia liberated in 1 hour was increased in the presence of any type of  $\alpha$ -amino acid or substitution product; there was a slight increase in the presence of  $\beta$ -amino acids.  $\gamma$ -Amino acids, amines, amides, uric acid, creatine, creatinine, guanidine, and ammonium chloride had no effect.

The concentration of proteolytic products present as  $\alpha$ -amino acids, peptides, etc., in a cattle bone phosphatase preparation is suboptimal. A hydrolyzing solution of glycerophosphate containing 75 per cent of bone phosphatase extract has a concentration of  $\alpha$ -amino acids, etc., much closer to the optimum than one at 12.5 per cent. Accordingly the accelerant effect is greater at the higher concentration of enzyme. This is evidenced by the fact that the ratio of the reaction velocities, as measured by  $Q$ , is much greater than 6.0, the ratio of the enzyme concentrations. Thus in Preparation CBH-1-d, a dialyzed cattle bone phosphatase  $Q_{0.05}$  at 75 per cent was 13.8 times that at 12.5 per cent (Table II). The addition of glycine decreased the disproportionality between enzyme concentration and reaction velocity, but at an optimal concentration of glycine the reaction velocity at 75 per cent phosphatase was still 8.3 to 9.0 times as large as at 12.5 per cent.

*Influence of Magnesium on the Accelerant Effect of  
 $\alpha$ -Amino Acids*

The accelerating effect of magnesium ion has been noted by several investigators (19, 24-26). The optimal effect is obtained at the concentration of 0.01 to 0.001 M. Table I indicates the nature of this acceleration. Magnesium ion not only prevents a rapid decrease in the rate of hydrolysis of glycerophosphate as the reaction proceeds, but also increases the velocity with which the reaction starts.

In Table IV are shown the accelerating effects of glycine on the action of a cattle bone phosphatase, Preparation CBH-1-d, in the presence of an added optimal concentration of magnesium ion, 0.0045 M. Comparison of the reaction velocities for the same enzyme preparation at the same concentrations of glycine (Table II) shows that the presence of magnesium causes the greatest

percentage of acceleration at the low concentrations of glycine. Thus  $Q_{0.05}$  at 0.0003 M glycine is increased from 0.0017 to 0.0081, or 380 per cent, while that at 0.00625 M is increased 110 per cent, and at greater concentrations still less. This has the effect of making the zone of optimal activity much broader. Comparison of the reaction velocities at 12.5 per cent and at 75 per cent of enzyme shows that *in the presence of optimal concentrations both of magnesium and of glycine, the reaction velocity is directly proportional to the concentration of bone phosphatase.*<sup>1</sup> The influence of other magnesium concentrations was not studied.

TABLE IV

*Dependence of Reaction Velocity on Concentration of Glycine in Presence of an Optimal Concentration of Magnesium*

Dialyzed cattle bone phosphatase, Preparation CBH-1-d; temperature, 25°; concentration of added magnesium, 0.0045 M.

Concentration of added glycine	Reaction velocity, $Q_{0.05}$ , at enzyme concentration of		$Q_{0.05}$ at 75 per cent $Q_{0.05}$ at 12.5 per cent
	12.5 per cent phosphatase	75 per cent phosphatase	
M per l.			
0.0	0.0081	0.0583	7.2
0.00031	0.0095	0.0603	6.4
0.00094	0.0098	0.0580	6.0
0.0025	0.0098	0.0591	6.0
0.00625	0.0100	0.0584	5.8
0.025	0.0078	0.0485	6.2
0.050	0.0052	0.0383	7.4

The findings so far reported were found to apply to other bone phosphatases. As prepared by a 1:20 extraction, rat bone phosphatase usually showed a direct proportionality of reaction velocity to enzyme concentration. Glycine did not increase the activity of such preparations. After dialysis, however, these preparations showed acceleration by glycine (Table V). Apparently the usual rat bone phosphatase preparation contained a concentration of proteolytic products that was in the optimal zone, and the addition of 0.0025 M glycine had no further effect. The

<sup>1</sup> This was demonstrated for a series of concentrations from 12.5 to 75 per cent.

dialyzed preparation showed also a disproportionality between reaction velocity and enzyme concentration. The undialyzed Preparation RBH had a  $Q$  at 12.5 per cent of 0.0103 and one of 0.0638 at 75 per cent, or 6.2 as large as at 12.5 per cent. After dialysis for 24 hours against several changes of distilled water,  $Q$  at 12.5 per cent was 0.0041 and at 75 per cent, 0.0435, or 10.6 times as much. Dialysis for longer periods led to the precipitation of protein and yielded slightly active preparations; acceleration by glycine was demonstrable in these preparations also.

A lamb bone phosphatase prepared by a 1:10 extraction showed a reaction velocity,  $Q_{0.02}$ , at 12.5 per cent concentration of enzyme, of 0.0033 and one of 0.0415 at 75 per cent; the ratio was 12.6.

TABLE V

*Effect of Glycine on Activity of Rat Bone Phosphatase*

Preparation RBH; temperature, 25°. After dialysis for 2 days, there was a precipitate of protein which may have carried down enzyme.

Concentration of added glycine  <i>M per l.</i>	Reaction velocity, $Q_{0.02}$ , at 12.5 per cent concentration of phosphatase in		
	Undialyzed preparation	Preparation dialyzed for 20 hrs	Preparation dialyzed for 2 days
0 0	0 0103	0 0041	0 00008
0 00031			0 00027
0.0025	0 0107	0 0058	
0 0031			0 00046
0.0156			0 00063
0 0312			0 00049

When glycine and magnesium chloride were added to the hydrolysis mixtures so as to give concentrations of 0.00625 M and 0.009 M, respectively, the reaction velocities were as follows:

	12.5 per cent phosphatase	75 per cent phosphatase	Ratio of velocities
$Q_{0.02}$	0 0077	0 0473	6.1
$Q_{0.03}$	0 0049	0 0298	6.1
$Q_{0.04}$	0.0034	0.0211	6.1

Direct proportionality was established between enzyme concentration and reaction velocity in the presence of optimal concentrations of glycine and magnesium.

A dialyzed rat bone phosphatase, Preparation RBH-a, showed disproportionality between reaction velocity and enzyme concentration at 37.5°;<sup>2</sup> it also showed marked acceleration by glycine. When *d*-isoleucine and magnesium chloride were added to the hydrolysis mixtures so as to give concentrations of 0.0075 M and 0.0045 M, respectively, the reaction velocities,  $Q_{0.05}$ , (at 25°) were 0.0065 at 12.5 per cent concentration of phosphatase and 0.0393 at 75 per cent; the ratio of the velocities was 6.0.

*Mode of Acceleration of Bone Phosphatase Activity by  $\alpha$ -Amino Acids*

Sherman and his collaborators (9, 13, 14) considered that the increase in amylase activity in the presence of  $\alpha$ -amino acids might be due (a) to a combination of the acids with the reaction products, thus neutralizing their retardant action; (b) to a neutralization of some toxic agent which destroyed the enzyme in the course of the reaction; (c) to a specific activating effect by the  $\alpha$ -aminocarboxy linkage; (d) to the fact that the enzyme, presumably a protein, was hydrolyzable in solution and that the  $\alpha$ -amino acids, as products of proteolysis, retarded such hydrolysis.

The experiments of Sherman and his collaborators favored the last of these possibilities. They found that the loss of activity which pancreatic amylase underwent rapidly on standing was prevented by the addition of  $\alpha$ -amino acids and that the extent of this stabilizing effect paralleled the known instability of the enzyme at higher temperatures. Rockwood (7) kept urease at the same temperature and pH as that of the hydrolysis for varying intervals of time before initiating the reaction. He found that the destruction of enzyme which occurred on standing under these conditions could be prevented by the presence of an  $\alpha$ -amino acid, but that, in addition, the  $\alpha$ -amino acid exerted "a specific stimulating action" during the course of the reaction. Similar results were obtained with salivary amylase.

According to the work of the above investigators, the effect of  $\alpha$ -amino acids on amylase and urease may be considered more accurately as the prevention of an inactivation rather than as a direct stimulation. In line with this concept it is also possible to

<sup>2</sup> The values for the reaction velocities at 25° were not available in this instance; however, it was found that a preparation which showed disproportionality at 37.5° also showed it at 25°.



consider a "specific stimulating action" during the course of the enzymic reaction as the prevention of a greater inactivation of the

TABLE VI

*Effect of Glycine on Inactivation of Bone Phosphatase at 25°*

Both were cattle bone preparations; concentration, 12.5 per cent. Preparation CBG was run at room temperature (24.5–25.5°). Each hydrolysis listed under "Preparation CBG" represents the optimal one of four incubated and reacting in the neighborhood of pH 9.0. For Preparation CBF incubations and hydrolyses were adjusted with the amount of NaOH necessary for optimal pH. Concentration of glycine in hydrolysis mixture, 0.00625 M.

Preparation CBG				Preparation CBF			
Time of hydrolysis	P liberated as phosphate per cc. of hydrolysis solution after incubation for			Time of hydrolysis	P liberated as phosphate per cc. of hydrolysis solution after incubation for		
	0 hrs.	22 hrs.	69 hrs.		0 min.	144 min.	340 min.

(a) Incubation and subsequent hydrolysis in absence of glycine

hrs.	mg.	mg.	mg	min.	mg.	mg.	mg.
0				0			
12	0 020	0 015	0 012	80	0 010	0 011	0 011
22	0 028	0 023	0 019	144	0 017	0 018	0 018
48	0 046	0 042	0 034	240	0 027	0 028	
69	0 054	0 051	0 044				

(b) Incubation and subsequent hydrolysis in presence of glycine

0				0			
12	0.033	0.028	0.023	80	0 019	0 019	0 017
22	0.048	0 043	0 035	144	0 031	0 030	0 026
48	0.073	0.068	0.057	240	0.045	0.044	
69	0 089	0.079	0.071				

(c) Incubation in absence, subsequent hydrolysis in presence of glycine

0				0			
12	0.034	0.023	0.016	80	0.019	0 020	0.017
22	0 049	0.035	0.022	144	0 031	0 031	0.028
48	0.075	0.056	0.038	240	0.046	0.044	
69	0.088	0 066	0.048				

enzyme in the presence of the substrate than in its absence (other conditions being the same). It is from this point of view that the

results on bone phosphatase are presented (Table VI). The justification for taking this view will be discussed more fully later.

Solutions of buffer, acid or alkali, and bone phosphatase in the same proportions as for the usual hydrolysis were prepared and kept at room temperature (24–25°) or in the thermostat at 25°, with and without glycine. Immediately after preparation of these mixtures and at stated times thereafter, 7 cc. were withdrawn and added to 1 cc. of 3.12 per cent solution of sodium  $\beta$ -glycerophosphate. To obtain controls for the effect of glycine, 6.9 cc. of the solutions containing no glycine were added, after incubation for a given period, to 1 cc. of the glycerophosphate solution and 0.1 cc. of 0.5 M glycine. The results of these experiments are given in Table VI; the values listed have been taken from curves constructed from the original data.

Preparation CBG, a weak cattle bone phosphatase, showed inactivation on incubation at room temperature ((a) Table VI). The presence of 0.007 M glycine in the incubating mixture greatly retarded this inactivation ((b) and (c) Table VI). Thus the mixture of buffer, enzyme, water, and  $\text{OH}^-$  ions at the optimal pH, incubated for 69 hours in the presence of glycine, liberated on addition to the substrate 0.023 mg. of phosphorus per cc. in 12 hours, 0.035 mg. in 22 hours, 0.057 mg. in 48 hours. In a mixture incubated for the same period in the absence of glycine (but to which glycine was added for control purposes at the initiation of the hydrolysis) 0.016 mg. of phosphorus per cc. was liberated in 12 hours, 0.022 mg. in 22 hours, and 0.038 mg. in 48 hours.

The inactivation which occurred during hydrolysis in the absence of glycine was, however, greater than could be accounted for by assuming that the inactivation was due merely to contact of the enzyme with buffer,  $\text{OH}^-$  ion, and water. In the presence of glycine 0.049 mg. of phosphorus per cc. was liberated in 22 hours; in the absence of glycine 0.028 mg. was liberated. The failure of the difference, 0.021 mg., to be hydrolyzed may be taken as a measure of the inactivation of the enzyme during the reaction due to the absence of glycine. When the enzyme was first incubated for 22 hours in the absence of glycine, then allowed to act on the substrate in its presence, 0.035 mg. was liberated per cc. in 22 hours of hydrolysis. The difference between this value and 0.049

mg., or 0.014 mg., is a measure of the inactivation during contact with buffer,  $\text{OH}^-$  ion, and water for 22 hours. This inactivation is less than the inactivation (failure to liberate 0.021 mg.) during the 22 hours of the hydrolysis.<sup>3</sup>

The greater inactivation which takes place during the reaction was illustrated more sharply in the case of the more active bone phosphatase, Preparation CBF. This preparation did not undergo any decrease in activity on incubation with buffer, etc., during 144 minutes, or any marked decrease during 340 minutes. In a hydrolysis in which glycine was absent, 0.011 mg. of phosphorus was liberated per cc. in 80 minutes, 0.018 mg. in 144 minutes, and 0.028 mg. in 240 minutes. In a hydrolysis in which glycine was present, the liberation at the end of these periods was, respectively, 0.019, 0.030, and 0.044 mg. In short, the inactivation of the enzyme, due to the absence of the glycine, was such that it failed to liberate 0.008 mg. in 80 minutes, 0.012 mg. in 144 minutes, 0.016 mg. in 240 minutes. This inactivation during the course of the hydrolysis cannot be accounted for by inactivation due to contact with water, buffer, etc., during the periods stated.

#### DISCUSSION

Nord (27) has stressed the view that so called activators are really protectors and "*enable the enzymes to act under conditions which are more nearly those which might be expected to be prevalent in ideal cases.*"<sup>4</sup> Sumner and Hand (28) applied this concept in the instance of crystalline urease. They showed that the inactivation which this crystalline enzyme underwent on standing could be prevented by a number of substances, including  $\alpha$ -amino acids, but that in no case did any of these protectors yield an enzymic activity greater than that of the not inactivated crystalline urease alone.

It has been shown in this study for bone phosphatase and by Rockwood (7) for urease and salivary amylase that the acceleration by  $\alpha$ -amino acids during hydrolysis is greater than can be

<sup>3</sup> Since, as pointed out previously, the amount of phosphorus liberated in a given time cannot be taken as a measure of phosphatase concentration, no attempt was made to express the inactivation in terms of per cent of enzyme inactivated.

<sup>4</sup> The italics are Nord's.

accounted for by the protective effect on the enzyme when not acting on the substrate. Rockwood (7) interpreted his findings to indicate a specific stimulating action in addition to the protective action during the course of the reaction. It is in accord with Nord's (27) concept and the work of Sumner and Hand (28) to consider that the greater accelerant effect of  $\alpha$ -amino acids during the enzymic reaction is merely indicative of a prevention of the greater inactivation taking place. Specific support for this view in the case of bone phosphatase is found from study of the course of glycerophosphate hydrolysis and of the relation between reaction velocity and enzyme concentration.

It has been shown how the presence of magnesium and an  $\alpha$ -amino acid prevents the rapid falling away in the velocity of hydrolysis. At suboptimal concentrations of magnesium and  $\alpha$ -amino acid a disproportionality exists between enzyme concentration and reaction velocity; at optimal concentrations a linear relationship between the two is established. If it is assumed that magnesium and  $\alpha$ -amino acids are, in the main, "specific stimulators," it follows that the establishment of the linear relationship between reaction velocity and enzyme concentration is purely fortuitous. This, however, seems very unlikely.

The rule that the velocity of an enzyme reaction is directly proportional to the concentration of enzyme preparation holds very widely. An exception to this rule (not due to faulty technique) was considered for a long time to exist in the case of pepsin; it was held that the reaction velocity was proportional not directly to the concentration of enzyme, but approximately to the square root thereof (Schutz' law). Northrop (23) showed, however, that this held only for insufficiently purified preparations of pepsin; products of proteolysis, present as impurities, combined at high concentrations of enzyme according to the mass law to form with the pepsin an enzymically inactive compound. The *active* concentration of enzyme was thus less than the *apparent*, and the reaction velocity was less than called for by the apparent concentration of enzyme. Purification of the pepsin prevented the formation of inactive compounds; the concentration of *active* enzyme was identical with the *apparent*, and the reaction velocity was directly proportional to either.

Northrop's differentiation between active and apparent concen-

tration of enzyme may be applied to bone phosphatase. The existence of a direct proportionality between reaction velocity and bone phosphatase concentration in the presence of optimal concentrations of magnesium and  $\alpha$ -amino acid would indicate *that all of the phosphatase is in an active form* (and, incidentally, fulfils Nord's view of protectors enabling the enzyme to act under more nearly ideal conditions). The rôle of the magnesium, since it increases the velocity with which the reaction starts, may then be considered as converting some inactive fraction of bone phosphatase to an active state; the rôle of the  $\alpha$ -amino acid may be considered as preventing the phosphatase from being changed into some non-active condition during the course of the hydrolysis.

Sherman and Walker (9) suggested that  $\alpha$ -amino acids prevented the inactivation of amylase by retarding its proteolytic hydrolysis. This question has not been studied in the course of the present work. Sumner and Hand (28) found that crystalline urease was inactivated in water distilled from a block tin apparatus, but not in water redistilled from glass or treated with hydrogen sulfide. They concluded that the inactivation was due to the destructive effect of some metallic ion, probably lead. In view of this finding, it is not possible to apply Sherman and Walker's suggestion generally.

Many studies have been appearing recently (29-33) on the change in the content of bone phosphatase and in the phosphatase concentration in other tissues during disease or following experimental procedure. It seems to the writer appropriate, whenever quantitative comparisons are made, to insure that the technique is such as to represent differences in the total concentration of enzyme, and not in that of substances which may affect the activity of the enzyme. The readiest criterion of the identity between the active and the apparent concentration is the applicability of the rule of direct proportionality between reaction velocity and enzyme concentration.

For the estimation of bone phosphatase, it is suggested that the reciprocal of the time necessary to produce a given change be used as velocity measure and that optimal pH, optimal concentrations of magnesium ion and of  $\alpha$ -amino acid be present. Such a procedure is practical and precise with crude extracts containing little phosphate, or with dialyzed extracts. The con-

ditions for the precise measurement of other tissue phosphatases remain to be elaborated. The method, however, has been indicated in the present work.

#### SUMMARY

1.  $\alpha$ -Amino acids in low concentrations, 0.0001 M to 0.01 M, accelerate the action of bone phosphatase.

2. The extent of the acceleration is a function of the concentration of  $\alpha$ -amino acid; at optimal concentrations, the extent is the same for different amino acids. The zone of optimal acceleration varies with the individual acid. At concentrations greater than optimal, the  $\alpha$ -amino acids exert a retardant effect.

3. Other organic nitrogen compounds, not possessing the  $\alpha$ -aminocarboxy linkage, do not accelerate hydrolysis. This is in agreement with the findings by previous workers on urease and amylase.

4. Direct proportionality between bone phosphatase concentration and reaction velocity is obtained when the concentrations of magnesium and  $\alpha$ -amino acids in the hydrolysis solutions are optimal. It is pointed out that, under these conditions, the concentration of active enzyme is identical with the apparent concentration.

5. The manner of acceleration by  $\alpha$ -amino acids is considered to be a prevention of the inactivation of the bone phosphatase which occurs during the course of the hydrolysis.

6. The significance of these findings in the estimation of the activity of bone phosphatase extracts is discussed.

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## DIFFERENTIAL QUANTITATIVE ANALYSIS OF BILE ACIDS IN BILE AND IN DUODENAL DRAINAGE

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Human bile contains a mixture of different types of bile acids, in which the cholic and desoxycholic acids predominate. These acids are largely combined with taurine and glycine to form the conjugated bile acids, glycocholic, taurocholic, glycodesoxycholic, and taurodesoxycholic acids. In addition to these bile acids, anthropodesoxycholic acid (chenodesoxycholic acid) and lithocholic acid may also be present. These different bile acids vary greatly in their biological properties. It would seem, therefore, of importance to have a quantitative bile acid method which would permit the differential analysis of these various acids. The methods commonly used at present do not attain this objective. By the Schmidt-Dart method (1) only the conjugated bile acids are determined; the free bile acids, which may be present in fairly large amounts, are not determined (2). The different modifications of the Pettenkofer (3) reaction tend to be inaccurate, since desoxycholic acid yields only one-half to two-thirds of the amount of color given by cholic acid (4). The Gregory-Pascoe reaction (5), however, is of great value, since it is specific for cholic acid. When used by itself, it is of little value in the analysis of human bile which contains a large proportion of desoxycholic acid. By the iron precipitation method of Szilard (6) and the modification by Peoples (7) the total bile acids are measured without differentiation. In addition the difficulties inherent in the variations in the solubility, precipitability, and molecular weights of the different bile acids present in the same sample are encountered. These same objections apply to methods based on surface tension (8),

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optical rotation (9), and certain biological properties (*e.g.*, the hemolysis of red blood cells (10)).

By the procedure described in this presentation an attempt is made to give a differential quantitative analysis of the predominating bile acids present in any one sample. There is failure to differentiate anthropolidesoxycholic and lithocholic acids present in human bile. Since these acids give no color with the Gregory-Pascoe reaction, they are included in the estimation of desoxycholic acid.

### *Method*

In order to arrive at a differential quantitative analysis of bile for bile acids it is necessary to obtain quantitative estimations of the bile acids conjugated with taurine and glycine, of cholic acid, of desoxycholic acid, and of free bile acids. These determinations are carried out on the same sample of bile as follows:

1. The percentage of bile acids conjugated with taurine and glycine, the total conjugated bile acids, is determined by the Schmidt-Dart method (see below).

2. The cholic acid is determined by a modification of the modified Gregory-Pascoe reaction (see below).

3. The total bile acids are determined by precipitation with ferric chloride (see below).

4. By subtracting the total conjugated bile acids from the total amount of bile acids, the percentage of free bile acid is determined.

### *Determination of Total Conjugated Bile Acids*

#### *Reagents—*

1. 95 per cent alcohol.
2. 0.5 per cent phenolphthalein (aqueous).
3. 8 per cent NaOH.
4. Nitric acid (concentrated acid diluted 1:2).
5. 30 per cent  $\text{NaNO}_2$ .
6. Acetic acid (glacial).
7. Nitric acid (concentrated).
8. Superoxol (30 per cent  $\text{H}_2\text{O}_2$ ).
9. Concentrated HCl.
10. Benzdine hydrochloride (4 gm. of benzdine dissolved in 50 cc. of 0.5 N HCl, made up to 250 cc. with water and filtered after standing overnight).

11. 0.2 per cent brom-thymol blue (100 mg. dissolved in 3.5 cc. of 0.05 N NaOH and made up to 50 cc.).

12. 0.2 N NaOH.

In determining the total bile acids conjugated with taurine and glycine, the method as outlined by Cuny (11) is followed in principle. 2 cc. of gallbladder bile, or 5 cc. of fistula bile, are measured out into a 150 cc. beaker and 80 cc. of alcohol are added. The beaker is immersed in a boiling water bath; the solution is brought to a boil and kept boiling for 5 minutes. The solution is then filtered through folded filter paper into a 100 cc. volumetric flask. The filter paper is washed twice with small quantities of 95 per cent alcohol. The volumetric flask is cooled and allowed to stand overnight. If the solution is clear and no precipitate is formed, it is made up to volume with alcohol. Two portions of 40 cc. each are measured out into 100 cc. beakers and the remainder is poured into a 50 cc. beaker.

If the alcoholic solution in the 100 cc. flask is opalescent, it is placed on the steam bath for  $\frac{1}{2}$  hour and allowed to stand for 1 hour, when a further small amount of protein will precipitate out. Refiltration is then carried out. The alcoholic filtrate is made up to volume, and the separate portions measured out as outlined above.

After these samples are dried on a water bath, one of the 40 cc. portions is transferred with water into a tube calibrated to 10 cc. and, after addition of 2 drops of phenolphthalein, is made just alkaline to this indicator with NaOH. The other 40 cc. portion is taken up with 5 cc. of 8 per cent NaOH and transferred to a special tube with a bulbous end, calibrated at 10 cc. A rubber stopper holding a thin capillary tube about 40 cm. long is placed firmly in the neck of this tube, which is then heated for 6 hours in a boiling water bath. After hydrolysis is complete, 2 drops of phenolphthalein are added and nitric acid (concentrated  $\text{HNO}_3$  diluted to 1:2) is added until the solution is acid. A few drops of NaOH are then added to make the solution just alkaline to phenolphthalein and the contents are made up to 10 cc.

3 cc. portions of both the hydrolyzed and non-hydrolyzed solutions are analyzed for amino nitrogen in a Van Slyke apparatus. The difference in amino nitrogen between the two solutions gives the total amount of amino nitrogen derived from the glycine and taurine of the conjugated bile acids.

The 20 cc. portion of the original alcoholic extract is dried and then heated on an electric hot-plate with 10 cc. of concentrated nitric acid. The beaker is covered with a watch-glass and several porcelain chips are added to prevent bumping. When the nitric acid has boiled down to about 1 cc., superoxol (30 per cent  $\text{H}_2\text{O}_2$ ) is added drop by drop and the heating continued. Altogether, about 5 cc. of superoxol are added. Heating is continued until the solution has boiled down again to about 1 cc., when the beaker is removed and the watch-glass washed down with water. The beaker is then dried on a steam bath and 10 drops of concentrated  $\text{HCl}$  are added. After 2 hours, 10 cc. of water are added, carefully washed down the sides of the beaker. Then 4 cc. of a saturated benzidine hydrochloride solution are added. After 10 minutes, 10 cc. of acetone are added and precipitation of benzidine sulfate is allowed to take place for half an hour. The contents of the beaker are then filtered through a 4G4 (Jena) glass filter, a suction bottle being used. The precipitate is washed several times with neutral acetone. The filter holding the precipitate is placed in a 250 cc. beaker containing about 50 cc. of boiling water brought to the neutral point by using brom-thymol blue as indicator. The contents are then titrated with 0.02  $\text{N}$   $\text{NaOH}$ , the final end-point being reached after boiling the solution again. The number of cc. of 0.02  $\text{N}$   $\text{NaOH}$  used in titration is multiplied by the factor 0.14. The resulting figure gives the amount of amino nitrogen derived from the taurine conjugated with the bile acids. The taurine amino nitrogen is subtracted from the total amino nitrogen and the remainder is considered to represent the amino nitrogen released from the glycine present. If the taurine amino nitrogen is found to be higher than the total amino nitrogen, the latter is considered to be derived wholly from taurine and is so calculated. The taurine amino nitrogen figure is multiplied by 29.14, and the glycine amino nitrogen by 28.29.<sup>1</sup> The results give the amount of bile acids conjugated with taurine and glycine respectively. Since the amino acids conjugated with cholic and desoxycholic acids cannot be separated, the figures are

<sup>1</sup> The factor, 29.14, is computed from the theoretical value, cholic acid + amino nitrogen. The factor, 28.29, is derived by dividing 29.14 by the empirical factor, 1.03, on account of the well known excessive amino nitrogen recovery from glycine by Van Slyke's method.

calculated on a basis of cholic acid. As a result there is a small error, since the molecular weights of these two bile acids are slightly different. The sum of the bile acids conjugated with glycine and taurine gives the total amount of bile acids conjugated.

### *Determination of Cholic Acid*

The Reinhold and Wilson (12) modification of the Gregory-Pascoe (5) reaction was found to be specific for cholic acid. However, the method had a drawback in that it was necessary to employ a color filter for the colorimetric reading. In addition, in the case of human bile, the reaction had to be carried out in the presence of a small amount of alcohol. These difficulties were overcome by combining the Reinhold-Wilson method with the procedure of Harwood (13) in which the proteins and pigments of bile are precipitated by zinc hydroxide. The clear aqueous extract of bile salts thus obtained can be used not only for the determination of cholic acid, but also as a means of estimating the total bile acids by an iron precipitation method (see below). In carrying out the Reinhold-Wilson procedure on human bile, it was found that off shade colors were often produced. The yellowish discoloration was traced to the presence of a fine precipitate of desoxycholic acid, which is present in relatively large proportions in human bile. This difficulty was overcome by the addition of alcohol to the reagents *after* the reaction had taken place.

#### *Reagents—*

1. 2 N KOH.
2. 40 per cent  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , which is titrated against 2 N KOH so that 10 cc. of  $\text{ZnSO}_4 = 10.8 - 11.2$  cc. of KOH, with phenolphthalein as indicator.
3. 2 N  $\text{K}_2\text{CO}_3$ .
4. 0.9 per cent furfural, made up on the day when used, from furfural kept in an ice box. The furfural is freshly distilled from commercial furfural, giving an almost colorless product. Fresh material should be redistilled every 3 weeks. The more colorless the furfural, the greater will be the amount of blue color produced by the Gregory-Pascoe reaction.
5. 16 N  $\text{H}_2\text{SO}_4$ , calculated from the specific gravity of the concentrated  $\text{H}_2\text{SO}_4$ ; thus 445.4 cc. of concentrated  $\text{H}_2\text{SO}_4$ , having a specific gravity of 1.84, are made up to 1 liter.

6. Cholic acid, Riedel-de Haen cholic acid, dried for 24 hours at  $110^{\circ}$ . 200 mg. are dissolved by adding 4.69 cc. of 0.1 N NaOH and made up to 100 cc.; from this solution standards containing 0.2 mg. and 0.4 mg. per cc. are made up as required.

1 to 2 cc. of human or canine gallbladder bile, 3 to 5 cc. of canine fistula bile, 10 to 30 cc. of human fistula bile, or 20 to 35 cc. of duodenal drainage material are the usual quantities measured out into a 50 cc. centrifuge tube. 3 cc. of 2 N KOH are then added and thoroughly mixed with the bile. When small quantities of concentrated bile are used, 15 to 20 cc. of water are added, the presence of alkali preventing the precipitation of protein. Following this, 3 cc. of 40 per cent zinc sulfate are added drop by drop with constant stirring. When the contents are thoroughly mixed, the supernatant fluid is separated by centrifuging and the precipitate washed three times with 15 cc. quantities of hot water. The four washings are poured into a 100 cc. volumetric flask. The precipitate is then washed three times with 20 cc. portions of 95 per cent alcohol. For the first washing cold alcohol is used, and for the subsequent washings hot alcohol. The alcohol washings are all transferred to a 100 cc. beaker and dried on a water bath. The small residue is taken up with 5 cc. of 2 N  $K_2CO_3$  and combined with the water washings from the 100 cc. beaker. A small amount of zinc carbonate precipitate will form, but this can be disregarded as its dry replacement is no more than 0.5 cc.

1 cc. is pipetted from the volumetric flask into a test-tube (16 mm. in diameter) and the procedure of Reinhold and Wilson is then followed. 1 cc. of 0.9 per cent aqueous furfural solution and 6 cc. of 16 N  $H_2SO_4$  are added and the tube heated for 8 minutes at  $70^{\circ}$  in a water bath. After cooling for 2 minutes the resultant blue color is compared with that of the standard. In the cases of human bile, 7 cc. of 95 per cent alcohol are added at this point to both the unknown and to the standard and thoroughly mixed before reading. The two standards, which are always prepared with the reading of each batch of unknown solutions, contain 0.3 and 0.4 mg. of cholic acid per cc. These solutions are made up from the Riedel-de Haen cholic acid which has been dried at  $110^{\circ}$  for 24 hours to drive off the combined alcohol present in this preparation. As a rule no more than four unknowns are prepared and read at one time, since the color tends to fade rapidly. If

the color of the unknown solution does not match in the colorimeter within 5 mm. of the reading of one of the standards, measured portions are removed from the 100 cc. flask and suitably diluted or concentrated. Since different samples of bile vary enormously in their content of cholic acid, this procedure gives a high degree of flexibility to the method.

Although it has not been found necessary to do so, the zinc carbonate precipitate may be removed from the extract by combining all the water washings in a 100 cc. beaker. This is evaporated down to 40 cc. and transferred to a 50 cc. centrifuge tube. 5 cc. of 2 N  $K_2CO_3$  are added and the tube heated for 15 minutes in a water bath. This causes the zinc carbonate to come down as a granular precipitate, which can be easily washed free of bile salts. The supernatant fluid is removed by centrifuging and poured into a 100 cc. flask as before. The precipitate is washed once with hot water. The alcoholic extract is then added to the volumetric flask, which is made up to volume. The result is a clear, colorless solution of bile salts.

It was found that the deviation from Beer's law was quite marked beyond a 30 per cent difference from the standard. Thus if 0.2 mg. is used as a standard and the colorimeter is set at 16 all readings beyond 11 or 21 tended to be markedly off the theoretical line and to give irregular results. Two standards were therefore used routinely. Any comparison which read above 0.5 mg. or below 0.15 mg. was rejected, and the unknown solution either diluted or concentrated appropriately.

As a rule the conjugated cholates, *i.e.* taurocholic and glycocholic acids, and most of the free cholic acid were removed by the water washings. It was to remove the glycocholic acid that hot water was used. Occasionally, especially in pathological bile, where the relative percentage of free cholic acid is high, some of this acid was present in the alcohol extract. It was for this reason that the alcohol extract was always added to the aqueous extract before determining the total cholic acid. Cold alcohol was used in the first alcohol washing to prevent too much desoxycholic acid from coming into the solution, since an excess amount of this acid prevented the pigment and zinc hydroxide from precipitating completely, probably as a result of the formation of a colloidal suspension.

Reinhold and Wilson's observations on the loss of color produced by cholic acid after it has been dried from an alcoholic solution was confirmed. In addition, heating cholic acid with a strong solution of alkali caused a large loss in its chromogenic properties. Thus 40 mg. of cholic acid were heated with 8 per cent KOH for 6 hours, and, after neutralization and drying, were extracted with alcohol. After the evaporation of the alcohol the cholic salt was taken up in water and estimated both by the Gregory-Pascoe reaction and by the iron precipitation method described later. By the first method there was a loss of 11.9 mg., or 30 per cent, while by the second procedure there was a recovery of 39.3 mg.,

TABLE I

*Recovery of Cholic Acid Added to Bile—Gregory-Pascoe Reaction*

Source of bile	Cholic acid found per cc.	Cholic acid added per cc.	Added cholic acid recovered
	mg	mg.	mg.
Dog fistula.....	0.160	0.325	0.316
" ".....	0.053	0.136	0.134
" ".....	0.114	0.066	0.063
" ".....	0.116	0.066	0.066
" ".....	0.123	0.066	0.064
" ".....	0.193	0.051	0.050
" ".....	0.165	0.051	0.053
Human duodenal drainage ...	0.330	0.200	0.203
" gallbladder.....	0.209	0.200	0.190
" ".....	0.131	0.200	0.188
" fistula . . . . .	0.310	0.200	0.190

or 99.3 per cent. Whether this loss is due to a steric or even structural change in the cholic acid molecule has not been determined as yet. It is for these reasons, therefore, that the precipitate is washed carefully with hot water and the aqueous extract heated as little as possible. The occasional small percentage of cholic acid present in the alcoholic extract leads, therefore, to a minimal loss of the color produced by the Gregory-Pascoe reaction. Table I illustrates the recovery of cholic acid added to bile, which is then precipitated and extracted in the usual manner. It can be seen that added cholic acid can be recovered within 6 per cent of its theoretical value; as a rule, the recovery value lies between 97 and 98 per cent.

Although cholic acid added to bile could be recovered within satisfactory limits, it was decided to investigate the effect of the presence of desoxycholic acid on the formation of color by cholic acid. This point is of special importance, since it was found that in human bile, unlike canine bile, the relative amount of non-cholic bile acids, presumably for the greater part desoxycholic acid, in the total bile acids is very high, running from 50 per cent (normal) to as high as 85 per cent or more. It was noted that, whereas extracts of dog bile gave clear blue colors with the Gregory-Pascoe reaction, human bile, especially pathological bile, often gave shades of blue discolored by a yellowish green tint. Although this discoloration was not noted by Reinhold and Wilson, since they used a color filter, they did find the presence of a turbidity in certain samples of pathological human bile.

To 0.2 and 0.4 mg. standards of cholic acid were added varying amounts of desoxycholic acid. These mixed bile acids were compared with standards of pure cholic acid after producing the blue color by the addition of furfural and sulfuric acid. The results are graphically presented in Chart 1. It was found that the discoloration in the blue color began to appear when the amount of cholic acid in relation to the whole amount of bile acids present fell below 33 per cent. On investigation it could be seen that under those conditions there was a faint opalescent appearance to the mixture of reagents in the tube owing to the finely suspended precipitate of the desoxycholic acid in the highly acid medium. As the ratio of desoxycholic acid rose, the opalescence increased and was responsible for the discoloration which made the use of a filter necessary in order to make the readings possible. The absorption of light by the very fine precipitate of desoxycholic acid made the readings somewhat higher than they should actually have been.

To overcome these difficulties it was decided to attempt to dissolve the precipitated desoxycholic acid. Since desoxycholic acid is soluble in alcohol, it was decided to repeat the previous experiments of reading pure cholic acid standards against the same standards to which varying amounts of desoxycholic acid had been added. After the tubes containing the reagents had been heated and then cooled for 2 minutes, 7 cc. of 95 per cent alcohol were added to each tube and the contents thoroughly mixed. The



turbidity and discoloration immediately disappeared and colorimetric comparisons became very easy to read. It was found, however, that the cholic acid in association with desoxycholic acid gave slightly higher readings than the cholic acid standards; the

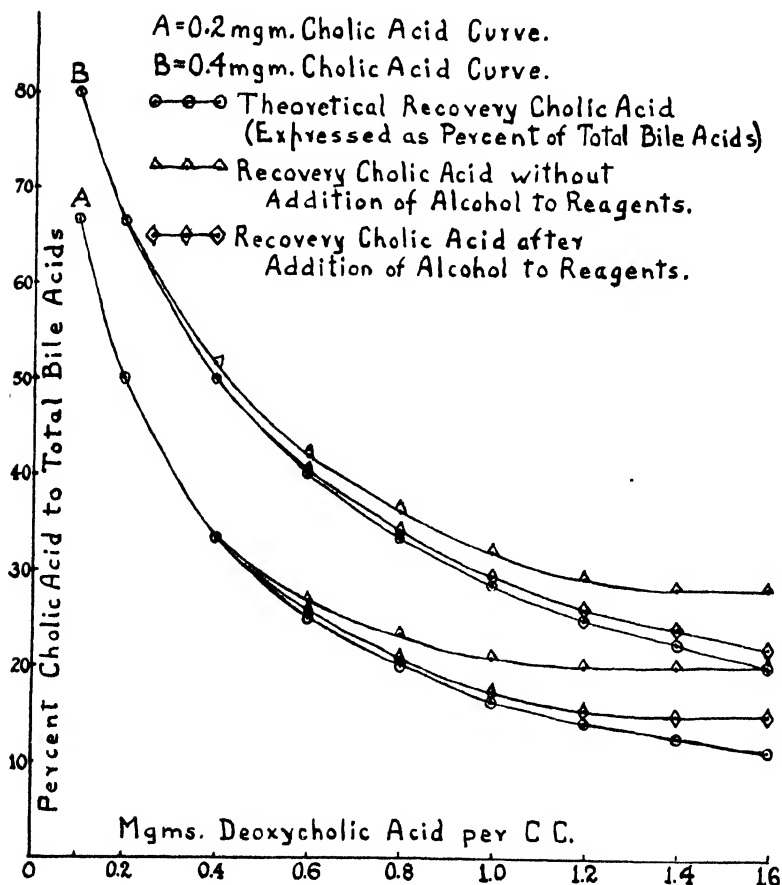


CHART 1. Recovery of cholic acid in the presence of desoxycholic acid

results are expressed in Chart 1 in the form of a curve giving the percentage of cholic acid to total bile acids. For each standard solution of cholic acid the lower line gives the theoretical percentage, the upper line the actual recovery if no alcohol is used, while

the middle line gives the recovery of cholic acid if alcohol is added to the reagents after the development of the color. This last line begins to diverge at 30 per cent for the 0.3 mg. standard and at 40 per cent for the 0.4 mg. standard. The divergence is small under these conditions even at the lower limits of the curve and indicates that when alcohol is added to the reagents after the reaction is completed, the Gregory-Pascoe reaction is for practical purposes specific for cholic acid. Whether the slight increase in color when desoxycholic acid is added is actually due to the reaction of this acid with the reagents in the presence of cholic acid, or whether there is a trace of cholic acid contaminating the Riedel-de Haen preparation of desoxycholic acid, has not been determined so far.

As a consequence of these investigations, it was decided to modify the procedure for the determination of cholic acid in human bile by adding 7 cc. of alcohol to both the standard and the unknown after the completion of color development. The readings could then be made with well matched colors and without the necessity of using a color filter.

### *Total Bile Acids*

#### *Reagents—*

1. 5 N  $\text{H}_2\text{SO}_4$ .
  2. Petroleum ether (b. p.  $35^\circ$ ).
  3. 100 per cent alcohol.
  4. N HCl.
  5. Superoxol (30 per cent  $\text{H}_2\text{O}_2$ ).
  6. 1 per cent phenol red.
  7. Ferric chloride solution, buffered (4 per cent  $\text{FeCl}_3$  and equal amount of borax-boric acid mixture).
  8. Borax-boric acid buffer. Boric acid solution (12.404 gm. and 2.925 gm. of NaCl made up to 1 liter), 950 cc., to which are added 50 cc. of borax solution (19.108 gm. made up to 1 liter).
  9. 0.5 N  $\text{Na}_2\text{SO}_4$ .
  10. Gum ghatti.
  11.  $\text{Na}_4\text{Fe}(\text{CN})_6 \cdot 12\text{H}_2\text{O}$ .
  12. 0.001 per cent  $\text{HgCl}_2$ .
  13. Pure iron wire.
- After the bile acids have been extracted and made up to 100 cc.

in a volumetric flask and the cholic acid determined, the amount of solution in the volumetric flask, estimated to contain between 40 and 60 mg. of bile acids, is measured out into a 150 cc. beaker. This calculation is derived from the known amount of cholic acid present in the volumetric flask. For this purpose the bile acids of dog bile may be considered to be approximately 100 per cent cholic acid, while those of human bile can be assumed to be 50 per cent cholic acid. Thus, if the volumetric flask is found to contain 100 mg. of cholic acid derived from dog bile, 50 cc. of the solution are measured in the beaker. If the extract is derived from human bile, only 25 cc. are taken.

The solution in the beaker is evaporated down to 30 cc. and 10 cc. of 8 N KOH are added. The beaker is covered with a watch-glass and hydrolysis is carried out on a boiling water bath for 6 hours. It is occasionally necessary to add a little water to maintain the volume.

After hydrolysis is completed, 5 N  $\text{H}_2\text{SO}_4$  is added until the solution becomes acid, *i.e.* the point at which the white cloud due to precipitated bile acids becomes permanent. About 10 cc. of 2 N  $\text{K}_2\text{CO}_3$  are then added to neutralize the excess  $\text{H}_2\text{SO}_4$ , and the contents of the beaker are brought to dryness on a water bath. The beaker then contains  $\text{K}_2\text{CO}_3$ ,  $\text{K}_2\text{SO}_4$ , bile salts, and any fats, cholesterol, etc., that have been carried over from the bile.

The beaker is dried for 20 minutes in an oven kept at  $110^\circ$  and the contents, after trituration, are extracted three times with 50 cc. quantities of low boiling ( $35^\circ$ ) petroleum ether. The petroleum ether is boiled down each time to half its volume. An electric hot-plate covered with a sheet of asbestos is used for this purpose. The petroleum ether is filtered through a small funnel holding No. 43 Whatman filter paper, any small particle rolling out of the beaker being caught by the filter paper. All the fats and cholesterol are extracted by this procedure; the potassium bile salts along with the  $\text{K}_2\text{CO}_3$  and  $\text{K}_2\text{SO}_4$  are left behind.

The beaker is allowed to stand until the residual petroleum ether has evaporated and is then placed in the  $110^\circ$  oven for 15 minutes. Absolute alcohol (15 cc.) is then added, and, after stirring, the alcohol is filtered into a 100 cc. beaker through the same funnel and filter paper that had been used previously. The contents of the beaker are extracted three times more with 15 cc. quantities

of alcohol, brought to the boiling point on a hot-plate. All the bile salts are extracted by this procedure, the  $K_2CO_3$  and  $K_2SO_4$  being left.

The alcoholic solution is evaporated to dryness on a steam bath, and the bile salts transferred with small quantities of water to a 15 cc. centrifuge tube, the total volume being about 9 to 10 cc. A drop of phenol red solution is used as indicator and 2 to 3 drops of 5 N  $H_2SO_4$  are added to neutralize any  $K_2CO_3$  that may have come through in the alcoholic filtrate. The centrifuge tube is then heated for 5 minutes in the boiling water bath to drive off  $CO_2$  formed. Just sufficient KOH is added to make the solution alkaline and to dissolve the precipitated bile acids. The contents, while still hot, are brought to a pH just below 7, the optimum point being where the pink color due to the phenol red just disappears. The centrifuge tube is cooled under the water tap and about 4 cc. of the ferric chloride solution, mixed with buffer, are added with a pipette. This results in a precipitate of ferric bile salts. After standing for 15 minutes, the contents are centrifuged and the supernatant fluid discarded. The precipitate is washed twice with 0.5 N  $Na_2SO_4$  to remove the excess ferric chloride. About 10 cc. of 2 N  $K_2CO_3$  are then added and the centrifuge tube heated for half an hour in the steam bath. After the material is centrifuged again, the supernatant fluid, containing the bile salts, is discarded and the precipitated ferric hydroxide is washed once with dilute  $K_2CO_3$  to remove any bile salts still present. The precipitate is then dissolved in about 10 cc. of N HCl and after 3 drops of superoxol are carefully added the centrifuge tube is placed in the boiling water bath for a half hour. The ferric chloride solution thus formed is transferred to a 25 cc. volumetric flask, which is made up to volume with N HCl. An appropriate amount, estimated from the color of the solution to contain about 0.5 mg. of iron, is transferred to another 25 cc. volumetric flask, and the determination of iron is carried out as outlined below.

#### *Determination of Iron*

The method used is based on a principle formulated by Harwood. The blue color produced by the ferrocyanide reaction with iron is kept in suspension by a solution of gum ghatti. The procedure, as modified and completed, is as follows:

*Preparation of Harwood's Iron Reagent*—Approximately 4 gm. of clear gum ghatti are placed in a copper wire basket suspended at the top of a 250 cc. measuring cylinder full of water. The gum ghatti is allowed to dissolve for 48 hours and the solution is then filtered twice through cotton. 2 gm. of sodium ferrocyanide ( $\text{Na}_4\text{Fe}(\text{CN})_6 \cdot 12\text{H}_2\text{O}$ ) are dissolved in 20 cc. of  $\text{N Na}_2\text{CO}_3$  and made up to 200 cc. with the gum ghatti solution. To this are added 2 cc. of a 0.001 per cent solution of  $\text{HgCl}_2$ . After the reagent is kept in the dark for 24 hours to allow impurities to precipitate, it is poured off into a dark bottle and is then ready for use.

*Preparation of Iron Standard*—200 mg. of pure iron wire are weighed accurately into a 50 cc. beaker and an equivalent amount of concentrated  $\text{HCl}$  is added. The beaker is placed on a boiling water bath until the iron is completely dissolved and 1 cc. of superoxol is then added. Heating is continued until no more bubbles are formed, and the solution transferred to a liter volumetric flask and made up to volume with  $\text{N HCl}$ . This stock solution, containing 0.2 mg. of  $\text{Fe}$  per cc., keeps indefinitely.

To make up the solution used as a standard, 50 cc. of the stock solution are transferred to a 100 cc. beaker and 10 drops of superoxol added. After being heated on a boiling water bath for 1 hour, the solution is transferred to a 200 cc. volumetric flask and made up to volume with  $\text{N HCl}$ . 10 cc. of this ferric chloride solution, containing 0.5 mg. of ferric ions, in the form of ferric chloride, are used as the standard.

*Procedure*—10 cc. of the iron standard are measured into a 25 cc. volumetric flask, and the unknown iron solution is placed into another 25 cc. volumetric flask, an amount to contain approximately 0.5 mg. of iron being used. To each flask are added 2 cc. of the iron reagent, and the contents are made up to volume with  $\text{N HCl}$ . The deep blue colors, which remain permanent for at least an hour, are read against each other in a colorimeter, the standard being set at 15 mm. This color reaction obeys Beer's law to very wide limits, although as a rule the unknown solution matches very closely to the standard, since the amount used can be easily determined, after some experience, from the intensity of color of the ferric chloride solution.

If any ferrous ions are present, even in traces, a greenish tint is present in the deep blue color of the reaction.

From the colorimetric readings, the total percentage of iron combined with the bile acids can be determined. Thus, 5 cc. of bile are extracted and made up to 100 cc., and 40 cc. of this volume are used for extraction. If 10 cc. of the eventual ferric chloride solution in the 25 cc. flask are used and the colorimeter readings are 15 (standard) to 10 (unknown), the calculation is as follows:

$$\frac{15}{10} \times \frac{0.5}{1} \times \frac{25}{10} \times \frac{100}{40} \times \frac{100}{5} = 93.75 \text{ mg. per cent Fe}$$

the calculation can be put in the form of a formula; thus, if

$a$  = reading of standard in colorimeter

$b$  = " " unknown " "

$c$  = iron standard in mg.

$d$  = cc. ferric chloride solution removed from 25 cc. flask containing unknown

$e$  = cc. fluid removed for hydrolysis from 100 cc. flask containing bile salt extract

$f$  = cc. bile taken for extraction

$$\frac{a}{b} \times \frac{c}{1} \times \frac{25}{d} \times \frac{100}{e} \times \frac{100}{f} = \text{mg. per cent Fe}$$

Thus from the above example, if the cholic acid is found to be 1.0 per cent, the equivalent amount of iron precipitated by it is  $1000 \times 0.0454 = 45.4$  mg. per cent of Fe (since 1 mg. of cholic acid combines with 0.0454 mg. of Fe). This is subtracted from the total iron percentage, leaving 48.35 mg. per cent of Fe, which is considered to be combined with the remaining non-cholic acids, and calculated as desoxycholic acid. Since 1 mg. of desoxycholic acid combines with 0.0474 mg. of Fe, the percentage of desoxycholic acid is  $48.35/0.0474 = 1020$  mg. per cent = 1.020 per cent. Adding the desoxycholic acid (1.020 per cent) to the cholic acid (1.00 per cent) gives the figure for the total bile acids (2.020 per cent).

#### *Calculation of Free Bile Acids*

Since the conjugated bile acids are calculated as bile acids conjugated with taurine and glycine, the sum of these is merely subtracted from the total bile acids. The resulting figure represents the free bile acids. The free cholic and desoxycholic acids cannot be differentiated at present.

*Recovery by Iron Precipitation Method of Pure Bile Acids Added to Bile*—Various amounts of cholic and desoxycholic acid were added to samples of bile of known salt composition, and the recovery of these added acids found by subtraction. It was found

TABLE II  
*Recovery of Bile Acids Added to Bile—Iron Precipitation Method*

Source of bile	Bile acids in sample	Bile acid (cholic acid) added	Bile acids found	Bile acid recovered
	mg.	mg.	mg	mg.
Duodenal drainage. . . . .	18 2	40	58 2	40
Gallbladder (human) . . . .	91 04	20	110 04	18 60
Duodenal drainage . . . . .	81 60	20	100 76	19 16
“ “ . . . . .	27 45	20	46 05	18 60
“ “ . . . . .	27 45	20*	46 45	19 00

\* Desoxycholic acid.

TABLE III  
*Typical Examples of Differential Bile Acid Analysis*  
The results are given in per cent.

Source of bile	Conjugated bile acids			Cholic acid	Desoxycholic acid	Total bile acids	Free bile acids
	With taurine	With glycine	Total				
Human gallbladder. . . . .	2 646	3 507	6 153	3 333	4 328	7 661	1 508
“ “ . . . . .	3 267	5 062	8 329	4 255	5 496	9 751	1 422
Canine “ . . . . .	9 553	0	9 553	10 416	3 572	13 988	4 435
“ “ . . . . .	7 533	0	7 533	8 820	2 566	11 386	3.853
“ “ . . . . .	8.101	0	8 101	9 132	3 559	12 691	4.590
Human fistula . . . . .	0.912	0 288	1 200	0 626	0 881	1 717	0.517
“ “ . . . . .	0.615	0 345	0 960	0 393	0 850	1 243	0 283
Canine “ . . . . .	1.645	0	1 645	2 110	0 649	2 759	1 114
Duodenal drainage Bile A.				0 129	0 118	0.247	
“ “ “ “				0 362	0 362	0 724	
“ “ “ B.				2 024	2 280	4 304	
“ “ “ “				0 521	0 940	1.461	

that both cholic and desoxycholic acid could be recovered to within 7 per cent of the theoretical value. As a rule the recoveries were much better. Some of the results are given in Table II.

*Typical differential bile acid analyses* of bile, of both human

and canine origin, are given in Table III. It will be seen that the conjugated acids are given as bile acids conjugated with taurine and glycine. This is done for the sake of convenience in estimating the free bile acids. The weights of the taurine and glycine are left out, however. To estimate the actual percentage of taurocholic acid, the figures in the first column should be multiplied by the factor 1.26. For the percentage of glycocholic acid, the figures in the second column should be multiplied by the factor 1.14. Thus, in the first examples of human gallbladder bile in Table III the actual percentage of total bile acids is as follows:

	per cent
Taurocholic acid = $2.646 \times 1.26$ .....	3.334
Glycocholic " = $3.507 \times 1.14$ .....	3.998
Total conjugated bile acids.....	7.332
Free bile acids. . . . .	1.508
Total " " .....	8.840

*Observations Relating to Methods Used*—In the determination of the conjugated bile acids it is necessary that all protein in the bile be precipitated. It was found that in certain cases, even a 15-fold dilution with boiling alcohol was not sufficient to cause precipitation of all the protein material. On allowing the alcoholic filtrate to stand overnight, a further precipitation of protein material occurred in a number of cases. In other cases the alcoholic filtrate was found to be opalescent; on further heating a precipitate appeared. These phenomena were invariably present in bile from pathological cases such as that removed from acutely inflamed gallbladders or in the fistula bile in cases of cholangitis. It was therefore found necessary to allow all alcoholic filtrates to stand overnight and to refilter them if a precipitate was present.

Even when all these precautions were taken the total conjugated bile acids, as estimated from the conjugated amino nitrogen, were often found to be greater than the total bile acids, as determined by other methods. Such results were found only in samples of bile removed from inflamed gallbladders or from the common duct the first few days after operation. This excessive nitrogen must owe its source to amino nitrogen-containing material soluble in alcohol.

It was found impossible to determine the conjugated bile acids in duodenal drainage material, since the pancreatic enzymes



acted on the protein material present with such rapidity that the broken down protein products soluble in alcohol invariably gave a result of conjugated bile acids much higher than the total bile acids actually present. This divergence in results increased the longer the duodenal drainage material was allowed to stand.

In the determination of taurine by the sulfur method, the results are undoubtedly higher than the amount of taurine actually present, since there is present a small quantity of other sulfur compounds, such as the ethereal sulfates. In normal human bile this error is negligible, but where the percentage of conjugated bile acids is very low, as in different types of pathological bile, the amount of amino nitrogen calculated from the sulfur content will often be found higher than the total amino nitrogen. This results in the masking of any glycine present. It has been our routine procedure to calculate the total amino nitrogen in such cases as derived from taurine with a full realization, however, that some glycine is most likely present. In canine bile the total amino nitrogen, as determined by the Van Slyke method, is used as a basis for the calculation of the acid conjugated; the conjugated amino nitrogen, as determined by the sulfur method, is always slightly higher than the amino nitrogen found by the Van Slyke method. This must, of course, be explained by the presence of sulfur compounds other than taurine.

In the determination of total bile acids by ferric chloride precipitation, it was found that the optimum amount of bile salts to be used for precipitation was between 30 and 60 mg., if a 15 cc. centrifuge tube was being used. Below that amount the bile salts tended to precipitate rather poorly; while, if over 60 mg. of bile salts were present, the volume of the precipitate tended to become inconvenient.

Although in the eventual extraction of the bile salts from the mixture of potassium sulfate and potassium carbonate absolute alcohol is used, the absorption of moisture from the air enables a slight amount of these latter salts to dissolve and pass through the filter. It is for this reason that the bile salt solution is acidified before precipitation with ferric chloride. The potassium carbonate is replaced by potassium sulfate and so does not interfere with the results. Potassium salts are used because  $K_2CO_3$  and  $K_2SO_4$  dry easily without water of crystallization, and are insoluble in absolute alcohol.

Since desoxycholic acid precipitates very readily below pH 7, it was very difficult to bring a mixture of cholic and desoxycholic acids to the acid side without causing a precipitation. It was eventually found that if the solution is kept hot the mixture could be brought to a pH just below 7 without producing any precipitation. Before adding the ferric chloride this solution could be cooled under the water tap without precipitation occurring.

Owing to their adsorptive qualities, bile salts tend to form a concentrated layer on the surface. The sudden addition of an acid solution of ferric chloride to this surface tends to precipitate a small amount of the bile acids before they can combine with the iron. It was found that if a borax-boric acid buffer with pH 6.95 was added to the ferric chloride solution the precipitation of free bile acids would not occur.

The ferric bile salt compound was found to be slightly soluble in distilled water. In addition, it tended to form a colloidal suspension in water. The same thing occurred if slightly acidified water were used. It was eventually found that a 0.5 N sodium sulfate solution prevented the formation of a colloidal suspension and in addition had a sufficiently low specific gravity to prevent the iron bile salts from floating to the surface.

In the determination of iron the greatest difficulty was encountered in preventing the formation of ferrous ions. The slightest trace of such ions produced a green color with the iron reagent and prevented an accurate colorimetric reading. Even the ferric chloride standard solution tended to acquire some ferrous ions. The simplest method of overcoming this difficulty was to add small amounts of superoxol to the ferric chloride solutions, to act as an oxidizing agent, and to change any ferrous ions present to the ferric condition.

#### SUMMARY

1. A method is presented for the differential quantitative analysis of bile acids in bile and in duodenal drainage material.

2. This method permits the analysis of bile for taurocholic acid, glycocholic acid, total conjugated bile acids, cholic acid, desoxycholic acid, total bile acids, and free bile acids.

3. This method permits the analysis of duodenal drainage material for cholic acid, desoxycholic acid, and total bile acids.

The author wishes to express his indebtedness to the late Dr. R. U. Harwood for many of the ideas and suggestions incorporated in this presentation.

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## INORGANIC SALTS IN NUTRITION

### CHANGES IN KIDNEYS OF RATS FED A DIET POOR IN INORGANIC CONSTITUENTS\*

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The correlation of kidney enlargement with various types of dietary unbalance has been well established. Nutritional abnormalities, as indicated by an excessively large proportion of protein, mere traces of inorganic salts, inadequate protein, or deficient vitamins, are known to stimulate growth of the kidney (Addis, MacKay, and MacKay, 1926-27, *a*; Winters, Smith, and Mendel, 1927; Osborne, Mendel, Park, and Winternitz, 1926-27; Jackson and Carleton, 1923).

Why diets so strikingly different induce growth of renal tissue, in most instances unattended by kidney damage, has not at the present time been determined. In the case of high protein feeding a work hypertrophy concomitant with the excretion of large quantities of nitrogenous waste products seems a reasonable explanation. However, MacKay, MacKay, and Addis (1931) cast doubt on as simple an answer to the question as this by demonstrating that quantities of protein and urea equivalent in nitrogen content do not produce equal kidney enlargements. There is little doubt that the renal growth, observed in rats largely deprived of inorganic salts, is associated with the necessary metabolic adjustment caused in turn by the dietary deficiency. Changes in chemical composition of the kidney can be expected to lead to a better understanding of the functional change pro-

\* A preliminary report was presented before the meeting of the American Physiological Society at Montreal, April, 1931.

duced in this organ by the specific nutritional alteration employed. The present study was, therefore, planned to determine whether the kidney enlargement first described by Winters, Smith, and Mendel is part of the integral adjustment (Swanson and Smith, 1932, *a*) of the mature as well as of the young animal to a paucity of dietary salts and whether increased size is associated with changes in composition of the kidney.

#### EXPERIMENTAL

*Plan*—70 albino rats, 37 days old, were given a purified ration extremely poor in inorganic constituents (Diet 3). One group of animals, twenty-five in number, was fed the diet for a period of 90 days, at the expiration of which time the animals were anesthetized, samples of blood for analyses obtained in a manner already described (Swanson and Smith, 1932, *a*), and appropriate tissues removed. The kidneys were extirpated immediately, trimmed carefully to the hylum, cut in half, and blotted once on filter paper. They were weighed at once, then dried to constant weight. Ash, calcium, and phosphorus analyses were made on the dried tissue.

In order to trace the influence of this particular dietary régime on the kidney, the organs were also removed from one group of rats that was killed at the initiation of the experiment, *i.e.* when 37 days old; from another group, after 21 days of experimental feeding (Diet 3); and from a third and fourth group after 42 and 63 days, respectively. The kidneys from each group of animals were compared with each other and with those removed at identical time intervals from 69 rats fed *ad libitum* the same low salt diet fortified with an adequate salt mixture (Diet 1).

Inasmuch as one of the effects of the feeding of salt-poor food is an immediate restriction of growth resulting finally in a maintenance of body weight at a constant level, a second control group was given the adequate synthetic diet until a body weight was attained equal to the average weight (160 gm.) of the group fed the ash-free ration for 90 days; they were then sacrificed. These animals were designated as the "physiological control" group. Only the weight of the kidney and its composition in regard to water and total solids were determined for this last group.

The necessity for the observance of certain precautions in the

selection of the experimental animals has been emphasized (Swanson and Smith, 1934).

*Diets*—The composition and preparation of the experimental diets have been described elsewhere (Swanson and Smith, 1932, a). When ashed with calcium acetate (Shaw, 1920) so as to prevent the loss of volatile acid anhydrides, the low ash diet contained 1.87 per cent of ash residue. The quantity of diet consumed by the rat daily with the vitamin adjuvants furnished approximately 66 mg. of ash. The ration has been shown to be extremely poor in base and potentially acidic in character (Brooke and Smith, 1933; Smith and Smith, 1934).

#### *Analytical Methods*

The fresh weight of the kidney was determined by weighing the organ immediately after extirpation in a covered bottle; moisture, by drying the kidneys to constant weight in an electric oven at 105°. The kidneys from each animal were ashed individually in platinum crucibles in a muffle furnace at approximately 540° after the addition of a carefully measured quantity (Shohl, 1928) of calcium acetate (Shaw, 1920). Calcium was determined by the Clark and Collip (1925) modification of the Kramer and Tisdall method from aliquots of an acid solution of the ash. The hydrogen ion concentration of the aliquot was adjusted with methyl red as the indicator (Shohl and Pedley, 1922). Phosphorus was determined by the volumetric molybdate method described by Hibbard (1913). The acid solutions of ashes obtained from each pair of kidneys, made up to a volume of 10 cc. and representing one-half of the kidneys of each group, were used for the analyses of calcium; the other half for the analyses of phosphorus. Both the calcium and phosphorus methods were accurate in our hands.<sup>1</sup>

#### *Results*

Rats consuming the adequate synthetic ration (Diet 1) grew at a rate equivalent to that of the stock colony (Swanson, 1930).

<sup>1</sup> Calcium per cc. of standard solution, 0.8869 mg.; average recovery, 0.8869 mg. (six analyses). Phosphorus per cc. of standard solution as checked by the Epperson (1928) method, 4.815 mg.; average recovery, 4.813 mg. (four analyses).

TABLE I  
Composition of Kidneys of Rats Fed Adequate Control Ration

Experimental period	Range of data	Weight of fresh kidney		Moisture		Total solids		Ash			Calcium				Phosphorus			
		No. of samples	gm.	No. of analyses	per cent	No. of analyses	mg	No. of analyses	On dry basis	Absolute value	mg. per gm	On dry basis	Absolute value	No. of analyses	mg. per gm	On dry basis	Absolute value	In ash
At beginning of experiment	Minimum	0	858		74.8		214											
	Maximum	1	167		77.5		282											
	Mean	12	1 012	12	76.5	12	238	14	6 1	14.5	0 16	0 036	0 051	5	0 20	0 20	2 28	13.1
	Minimum	1	061		74.8		256											
After 21 days	Maximum	1	492		77.7		333											
	Mean	12	1 253	11	76.4	11	294	21	5 8	16.4	0 16	0 044	0 026	10	0 16	0 16	1 72	10.6
	Minimum	1	219		74.1		315											
	Maximum	1	725		77.0		397											
" 42 "	Mean	8	1 496	12	75.6	12	366	6	5 9	22.3	0 17	0 059	0 031	3	0 17	0 17	2 42	11.4
	Minimum	1	519		73.8		343											
	Maximum	1	891		77.4		469											
	Mean	13	1 690	13	75.5	13	414	6	6 0	26.6	0 23	0 081	0 040	5	0 23	0 23	2 42	11.6
" 63 "	Minimum	1	891		73.8		343											
	Maximum	1	891		77.4		469											
	Mean	13	1 690	13	75.5	13	414	6	6 0	26.6	0 23	0 081	0 040	5	0 23	0 23	2 42	11.6
	Minimum	1	684		74.1		379											
" 90 "	Maximum	2	349		79.4		554											
	Mean	24	1 964	23	75.9	23	494	19	5 5	26.0	0 11	0 052	0 019	9	0 11	0 11	1 91	7.4

TABLE II  
Composition of Kidneys of Rats Fed Salt-Poor Ration

Experimental period	Range of data		Weight of fresh kidney		Moisture		Total solids		Ash			Calcium				Phosphorus			
			No. of samples	gm.	No. of analyses	per cent	No. of analyses	mg.	No. of analyses	(In dry basis	Absolute value	mg. per gm.	Absolute value	In ash	No. of analyses	On dry basis	Absolute value	In ash	
At beginning of experiment	Minimum	0.858		74.8	214					5.8	12.6	0.10	0.025	0.19		6.91	1.52	11.1	
	Maximum	1.167		77.5	282					6.6	17.4	0.20	0.051	0.36		8.56	2.28	13.1	
	Mean	1.013	12	76.5	238	14				6.1	14.5	0.16	0.036	0.26	7	7.49	1.79	12.1	
After 21 days	Minimum	1.374		75.2	325					5.1	14.9	0.26	0.096	0.36		6.19	2.36	11.2	
	Maximum	1.892		79.2	394					6.1	22.2	0.44	0.149	0.78		8.63	2.81	14.7	
	Mean	1.510	13	76.7	352	12				5.8	19.8	0.36	0.110	0.54	4	7.52	2.56	13.4	
“ 42 “	Minimum	1.347		77.4	295					5.7	17.7	0.16	0.051	0.24		6.77	2.34	10.4	
	Maximum	1.653		79.9	371					7.7	23.3	0.40	0.148	0.64		7.92	2.42	18.0	
	Mean	1.512	12	78.2	330	11				6.6	21.1	0.25	0.089	0.39	4	7.48	2.40	12.8	
“ 63 “	Minimum	1.010		76.6	225					5.8	14.4	0.19	0.058	0.22		6.79	2.24	10.5	
	Maximum	1.624		79.9	354					6.6	23.1	0.29	0.089	0.45		7.83	2.43	12.9	
	Mean	1.413	10	78.0	311	10				6.3	19.6	0.25	0.073	0.37	4	7.22	2.34	11.9	
“ 90 “	Minimum	1.107		78.0	225					5.3	12.1	0.10	0.038	0.15		6.64	1.85	11.8	
	Maximum	1.671		81.7	350					9.0	24.3	0.22	0.067	0.34		8.38	2.51	14.7	
	Mean	1.330	26	79.4	294	25				6.3	18.2	0.17	0.051	0.26	13	7.70	2.23	13.0	



Kidneys also showed a consistent increase in weight at each experimental interval (Table I). Each increment was fairly closely related to the simultaneous augmentation that had occurred in body size—a normal correlation (Donaldson, 1924). With dietary salts strictly limited, the orderly progressions in weight were not observed (Table II).

After 21 days of experimental feeding the kidney had grown to such an extent that the increment was twice normal. Weight was sustained at this level for the next 21 days, after which the kidney shrank so that at the end of 90 days it was only 32 per cent larger than it was in the beginning, whereas the normal kidney had increased by 94 per cent. In comparing the average weight of the kidneys of rats on the low salt diet with that of normal animals of the same size (the physiological controls) some increase in size

TABLE III  
*Kidney to Body Weight Ratios\**

Diet	Beginning of experi- ment	After 21 days	After 42 days	After 63 days	After 90 days
Adequate	8.4	6.1	5.8	5.6	5.6
Low salt	8.4	10.2	9.6	9.0	8.4

\* Divided by 0.001.

was noted; *i.e.*, 1.330 gm. *versus* 1.216 gm. (d./p.e.d., 5.1).<sup>2</sup> The irregular growth of the kidney in the rats fed the ash-poor diet created the distortion of the kidney to body weight ratio shown in Table III.

The early enlargement observed at the end of 21 days is evidently a characteristic response to the specific dietary restriction of this experiment, for it is to be recalled that the same phenomenon has been noted in young, newly weaned rats fed the deficient ration for 40 days (Winters, Smith, and Mendel, 1927; and Smith and Schultz, 1930). The regression in size following continued maintenance upon the salt-poor diet has not heretofore been recorded.

<sup>2</sup> In the ratio, d./p.e.d., d. is the difference between the means and p.e.d. is the probable error of the mean difference. If the value of the ratio is equal to 3.0, the difference in the two means may be considered significant; *i.e.*, the chances are greater than 20 to 1 that the difference is real.

In the normal rat, the average per cent of moisture decreased somewhat in the 90 day period (d./p.e.d., 2.6) and on the absolute basis, the quantity of total solids doubled. Absolute amounts of total ash, calcium, and phosphorus likewise increased. However, significant losses in the percentage of these constituents occurred before the termination of the experiment. Hogan and Nierman (1927) have also observed that the relative amount of inorganic salts in internal organs diminishes with age. The loss in concentration of both calcium and phosphorus was approximately 33 per cent. The percentage of calcium in kidney ash was constant until the last period; the relative quantity of phosphorus decreased at each experimental interval. The ratio of the per cents of the calcium and phosphorus in the ash was approximately 0.025, the values for the respective intervals of the experiment being 0.021, 0.028, 0.023, 0.029, and 0.026.

Inspection of the tables shows that the composition of the renal tissue in regard to the foregoing constituents was entirely different in the rats fed the ash-poor diet for 90 days. At the end of the experimental period, the most significant findings in relation to the normal picture were: a definite hydration of kidney tissue (d./p.e.d., 14.3), a high proportion of ash (d./p.e.d., 6.5), a high per cent of calcium (d./p.e.d., 8.23), and an increase in relative and absolute phosphorus. The abnormally large quantity of total solids present after 21 days of experimental feeding had decreased.

Data relating to each specific experimental period again showed a disturbance in the usual sequence of events. Abnormal changes in composition of the kidney were particularly pronounced after 21 days. Determinations of moisture, total solids, and per cents of ash and phosphorus indicate on first study that the marked kidney enlargement at this period was a simple hypertrophy brought about by new physiological demands (a conclusion similar to that reached by Smith and Schultz (1930) when they examined kidneys removed from young rats). However, the striking aspect of the picture in this instance lies in the marked increase in the calcium content of the tissue. Also, the per cent of calcium in the ash was doubled, resulting in a distortion of the proportion of calcium to phosphorus in the inorganic residuc. The calcium to phosphorus ratio of the increased ash after 21 days was 0.040 in contrast to the average value of 0.021 in ash derived from

kidneys of normal rats. Therefore, although certain of the data warrant the assumption of simple kidney enlargement, the new tissue does not have a normal composition. This period evidently represented one of critical change. It was followed closely by the sudden influx of erythrocytes into the blood stream (Swanson and Smith, 1932, b).

Several hypotheses may be advanced to explain the notable increase in kidney size. The renal changes observed may be related to the attempts of the animal to maintain acid-base equilibria in spite of the insufficiencies of the diet. A multiple strain has been placed on the organism by offering a diet not only deficient in the essential elements required for growth and well being but one also extremely poor in base, of potential acidity, and with a distorted Ca:P ratio (Brooke and Smith, 1933; Smith and Smith, 1934). Under these circumstances, the animal cannot afford to lose base in the neutralization of metabolic acids derived from dietary and tissue sources. The effort to conserve base, as shown by a higher urinary acidity, and to rid the tissues of excess phosphoric acid of dietary origin by an increased excretion of urinary ammonia, has been clearly demonstrated by Brooke and Smith (1933). If urinary ammonia, as Benedict and Nash (1929) conclude, is produced by the kidney, the definite increase in renal tissue observed in the first period of the experiment may be an expression of an attempt of the animal to manufacture sufficient ammonia for the excretion of excess phosphoric acid without undue loss of fixed base. It should be noted here that neither Addis, MacKay, and MacKay (1926-27, b) nor Osborne *et al.* (1926-27) were successful in producing kidney hypertrophy by feeding an adequate diet made potentially acid by the addition of ammonium chloride. Under the conditions of their experiments, however, as stringent a demand to conserve total base was not placed upon the organism as in the present study, where the physiological emergency according to Brooke and Smith (1933) is so great that the animal is actually able to produce more than sufficient base in the form of ammonia to neutralize metabolic acid for excretion in all but the last interval of the experiment. These authors further postulate that one of the causes of death in these animals is a failure in the ammonia production mechanism. The regression in kidney weight during the final period may indi-

cate such a breakdown in kidney function. In this connection, Osborne, Mendel, Park, and Winternitz (1926-27) suggest from data recorded by Hinman (1923) that an overwhelming demand may actually lead to atrophy of the kidney. Despite the unmistakable evidence of loss of renal substance between the 21st day of the period and the end of the experiment, histological examination<sup>3</sup> at 90 days indicated no structural abnormalities.

Another explanation may involve the vague relationship between the basal metabolic rate and renal enlargement. MacKay, Smith, and Closs (1933) have described hypertrophy of the kidneys in rats treated with desiccated thyroid, an enlargement which they believe is not dependent upon endogenous protein catabolism or excretion of excess urinary phosphate. Kriss and Smith (1935) have demonstrated that strict limitation of inorganic salts in the diet results in an elevation of the basal metabolic rate. It thus appears that energy metabolism exerts an influence on renal activity but in a way not at present evident.

No explanation can be offered at the present time for the elevated calcium and phosphorus content of the kidney after feeding the salt-poor food for 3 weeks. It might be noted in passing that the rise above normal in phosphorus, calculated as  $\text{Ca}_3(\text{PO}_4)_2$ , in these kidneys corresponded with the rise in ash, *i.e.* 4.2 and 3.4 mg. This suggests that at this stage of the experiment the physiological demand for calcium is being met by the mobilization of bone ash (Armsby, 1917), that the calcium thus released is utilized physiologically to a large extent, and that the phosphorus is excreted. Brooke, Smith, and Smith (1934) have demonstrated, however, that after 90 days on the low salt ration, a differential resorption of the components of bone ash has occurred. The withdrawal of calcium from bone carbonate may come in later stages of the experiment.

Similar increases in the concentration of ash, calcium, and phosphorus of renal tissue have been described by Morgan and Samisch (1935) following viosterol and parathyroid administrations. It may be that, as a result of the paucity of dietary calcium in the present study, a stimulation of parathyroid activity takes place.

<sup>3</sup> We are indebted to Dr. T. S. Moise for the histological examination of the kidneys.

An imbibition of water by the kidney follows the changes in the quantity of ash residue present. The increment in regard to this constituent is most marked at the end of the experiment, when the per cent of water is 79.4 in contrast to 75.8 per cent in the normal organ (d./p.e.d., 20.7). The high concentration of moisture in the kidneys of the rats on low salt diets may be a reflection of an attempt of the organism to maintain a satisfactory physiological ionic concentration of cells and interstitial fluids or may be due merely to retained urine. Other experiments have shown that these rats have a tendency to excrete urine in quantities somewhat above normal (Swanson, Timson, and Frazier, 1935). No dilatation of tubular epithelium was observed, however, in the present study.

#### SUMMARY

When inorganic salts are withheld from the diet of the albino rat, the emergency is met by an adjustment in various body systems. It has been shown in the present investigation that the excretory system is concerned. Soon after the initiation of the experiment, the kidney becomes definitely enlarged, the augmented weight being maintained for approximately 45 days. Then follows a regression in size, owing to a loss of total solids. The kidney is definitely hydrated when the experiment is terminated. The proportions of ash, calcium, and phosphorus do not decrease as they do under normal circumstances with advancing age. The kidney of the rats fed the ash-poor diet is particularly rich in calcium at the end of the first 3 week period; then the relative amount falls to the level present at the beginning of the experimental period. Phosphorus remains at the high level observed at the beginning of the experiment. The calcium to phosphorus ratio in the excess ash of the kidneys of the experimental rats is abnormal. The possible correlations of these data to metabolic findings already reported are discussed.

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## STUDIES ON THE ACID-BASE CONDITION OF BLOOD

### V. THE INFLUENCE OF PROTEIN CONCENTRATION ON THE COLORIMETRIC pH DETERMINATION OF BLOOD SERUM\*

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In the Cullen method (1922), or its various modifications, for the determination of the pH of blood serum or plasma, the sample is diluted 1:20 in physiological saline containing phenol red, and the color compared with appropriate standards. The *C* correction is the difference between the color reading of the diluted sample at 20° and the electrometric pH of the undiluted serum or plasma at 38° as determined by the hydrogen electrode. On diluting serum with saline containing dye, there is practically no further color change between the dilutions of 1:15 and 1:40 and therefore Cullen chose the 1:20 dilution as ideal. It was thought that under these conditions the salt and protein effects on the color of the indicator would be constant and negligible. That the dilution of 1:20 is the optimum dilution has been confirmed by other workers (Hastings and Sendroy, 1924; Austin, Stadie, and Robinson, 1925), and, with the present more exact technique, in this laboratory (unpublished data).

Experience with the method during the last 12 years has shown that the *C* corrections of sera, from any given species, are fairly constant. However, it has been observed by many workers, including ourselves, that at times there may be considerable variation in the *C* correction of a single serum. Repeated *C* determinations, made over a considerable period of time on apparently

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normal laboratory workers, indicate that the  $C$  correction remains constant within 0.02 pH, which is within the limit of experimental error. However, the variations which we have encountered in patients with various abnormal conditions (see Fig. 1 of this paper), those that have been reported by others, especially by Myers and Muntwyler (1928), and, further, the rather wide variation in individual dogs in our own experience, all emphasize the desirability of ascertaining the causes of these variations. This laboratory has been devoting itself for some time to an attempt to study the causes of these variations and this report concerns one phase of this study.

It has been stated at various times that no correlation exists between the magnitude of the  $C$  correction and the protein concentration in blood serum or plasma. Marrack and Smith (1924) obtained the  $C$  correction on nineteen pathological human plasmas. The pH of the undiluted plasma at 38° was calculated, from the  $\text{CO}_2$  tension and bicarbonate concentration, with the formula given by Austin *et al.* (1922). The protein concentrations were determined by the Wu method (1922). In fourteen determinations the results of Marrack and Smith showed a variation of total protein from 3.7 to 7.8 gm. per 100 cc. In every case the albumin-globulin ratio was greater than 1. The  $C$  corrections varied only from 0.20 to 0.27 pH and showed no direct variation with the protein concentration. They concluded that the correction in the Cullen method does not change with abnormalities of the plasma proteins. Similar conclusions were drawn for blood serum in the report of Cullen, Keeler, and Robinson (1925), in which the electrometric pH determinations were made in the Clark-Cullen hydrogen electrode vessel by a combination of Höber's gas mixture method and the Hasselbalch refill technique and the proteins were determined refractometrically. The serum protein concentrations varied only from 6.1 to 9.6 gm. per 100 cc. In Fig. 8 of this paper there is apparently no relationship between the  $C$  correction and the serum protein in twelve pathological human sera. Myers and Muntwyler (1928), for a much larger group of plasmas from hospital patients, state that in the variations of  $C$  corrections observed, there is "no indication that a change in protein concentration is a factor." For 103 samples, the average  $C$  correction was 0.224 pH, with maximum deviations from 0.14 to

0.30 pH. There were 51 protein color correction comparisons. The protein values were obtained from nitrogen determinations and electrometric pH determinations were made with the refill technique in Clark-Cullen vessels. Myers, Muntwyler, Binns, and Danielson (1933) in Table III of their paper present *C* corrections and serum proteins on thirty-nine samples from hospital patients. These results showed no correlation between the *C* corrections and the protein concentration. Their protein values, based on nitrogen determinations, vary in concentration from 5.0 to 7.6 gm. per 100 cc. and the *C* correction values from 0.22 to 0.36 pH, with an average of 0.298 pH. From these data it would appear that protein concentration is of little importance in the problem of variation in *C* correction.

On the other hand, the actual change in pH that takes place when serum at 38° is brought to 20° and diluted 1:20 in saline is caused partly by a shift in the equilibrium of the serum protein-carbonic acid system. Therefore, in sera of different protein concentrations, the actual change in pH on dilution and lowering of temperature would not be the same. Stadie, Austin, and Robinson (1925) have discussed the influence of this factor on the *C* correction. Hastings and Sendroy (1924) concluded that at 1:20 dilution the protein error of the indicator is negligible, but that deviations in the *C* correction may be accounted for as a result of variations in change in pH due to the temperature, depending upon the type and concentration of the protein. The experiments of Marrack and Thacker (1926) seemed to show that the protein error is not negligible at a 1:20 dilution. They diluted human plasma 1:20 in phosphate and bicarbonate buffer solutions containing phenol red and argued that, since the addition of 1 part of plasma to 20 parts of buffer solution does not apparently affect the actual reaction, any difference in color would be due to the effect of the plasma on the indicator. They found that the plasma of a nephritic patient, with a protein concentration of 4.6 gm. per 100 cc., reduced the color equivalent by 0.025 pH, whereas a normal plasma, containing 7.6 gm. of protein, reduced the color equivalent by 0.06 pH.

Our desire to restudy the protein effect was increased after we had accumulated data both on the *C* corrections and protein concentrations of human sera from normal individuals, from hospital

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patients with no disturbance in the acid-base condition, from nephritic patients in the terminal acidotic stage, and from nephrotic patients with low total serum protein concentration and a low albumin to globulin ratio. These data for 74 samples of human sera are given in Fig. 1. The points surrounded by circles indicate sera with a high total protein concentration but with an extremely low albumin to globulin ratio. Except for these latter determinations, there is a general tendency for the  $C$  correction

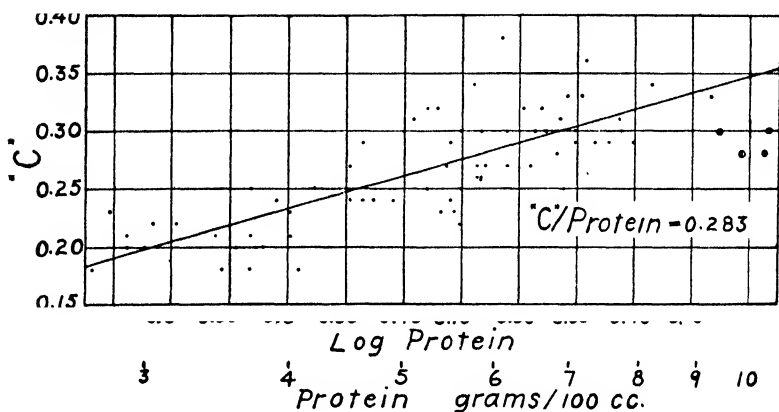


FIG. 1. The relation of  $C$  correction to protein concentration (plotted logarithmically) for normal human serum and for serum from hospital patients. All sera were equilibrated in a Simms electrode vessel at approximately 40 mm. of  $p\text{CO}_2$ . The encircled dots indicate sera with the unusual condition of a very high protein and extremely low albumin to globulin ratio. The straight line was fitted to the data by the method of least squares. The slope  $C/(\log \text{protein})$  for this line, 0.283, compares favorably with the average value of 0.243 that was obtained in the equilibrated dog serum experiments.

to increase with protein concentration. The reason that the tendency has been overlooked before is undoubtedly due, first, to the smaller range of variation of protein concentration in former series and, secondly, to other influences which may obscure the protein effect.

Although the observations, reported above, of Myers, Muntwyler, Binns, and Danielson on the pH of the sera of hospital patients showed no correlation between the  $C$  correction and the

protein concentration, their experiments with pooled human sera and pooled dog plasma did show a correlation. They varied the concentration of total protein in the sample by so diluting with salt solution that the salt concentration was maintained at a constant level. The solutions were then brought back to the same pH range by equilibration with CO<sub>2</sub> mixtures. They then found that in a constant pH range the *C* corrections decreased with protein concentration.

The average value of the *C* correction of normal human sera as determined with our technique is 0.30 pH, and the average value of the correction of sera from nephrotic patients is 0.23 pH (see Robinson, Price, and Cullen (1933)). Could the difference in *C* correction of 0.07 pH between our normal subjects and our nephrotic patients be accounted for by a difference in total protein concentration alone? We decided to compare the change in *C* correction with the change in protein of serum from a single sample of blood, varying the protein content by dilution and concentration of the serum. In order to obtain concentrated serum and also a suitable medium as diluent for the original serum, it was decided to resort to ultrafiltration. To avoid any influence due to change in total CO<sub>2</sub> content and pH, all samples were equilibrated at the same CO<sub>2</sub> tension. This allowed determinations on the original serum, diluted serum, and concentrated serum with constant CO<sub>2</sub> content and pH, and variation only in the protein concentration. In order to obtain adequate amounts of sera, the entire series of experiments reported here was carried out on dogs.

### *Technique*

*Ultrafiltration of Serum*—The ultrafiltration apparatus, made of Allegheny metal (Fig. 2), was modeled after the one described by Nicholas (1932). The filtering chamber is 3.8 cm. in diameter and has a capacity of about 40 cc. A feature of this apparatus is the top piece *D* (Fig. 2). When it is removed the filtering chamber is wide open and the contents may be stirred thoroughly with a glass rod. The tubing *A* (Fig. 2) connects to a high pressure oxygen cylinder. The serum was filtered under a pressure of 150 pounds per sq. inch through a cellophane membrane (du Pont No. 300). The ultrafiltrates were always clear, colorless, and protein-free by Heller's ring test. The concentrated serum always

appeared clear and uniform. The rate of filtration averaged about 0.5 cc. per hour. The rate of filtration decreases with time, as the serum near the membrane becomes very concentrated. This is shown by the following experiment. A sample of dog

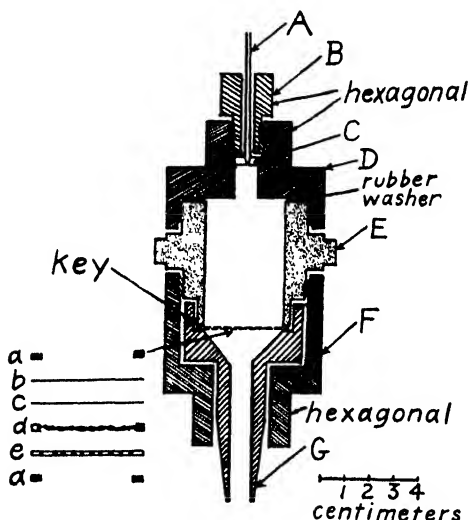


FIG. 2. Ultrafiltration apparatus. *A* is the  $\frac{1}{8}$  inch, outside diameter, flexible copper tubing connecting to the high pressure gas tank. It is brazed to the brass seat *C* which is held in place by the plug *B*. *D* is a removable top. The filtering chamber *E* and funnel *G* with the membrane between are held in place by the nut *F*. Parts *B*, *D*, *E*, *F*, and *G* are made of Allegheny metal. The key shown in *E* fits into a slot in *G* and prevents the shearing of the membrane on tightening *D* and *F*. The projections on *E* fit into a slot in a vise fastened to the laboratory bench that holds the apparatus during filtration and in the tightening and releasing of *B*, *D*, and *F*. The small letters at the side indicate the arrangement of the individual units of the filter disk. *a*, rubber washers; *b*, No. 300 du Pont cellophane membrane; *c*, No. 42 Whatman filter paper; *d*, 20  $\times$  20 mesh silver gauze mounted in an Allegheny ring; and *e*, perforated Allegheny metal plate.

serum with a protein concentration of 5.5 gm. per 100 cc. was placed in the apparatus under a pressure of 150 pounds per sq. inch for 7 $\frac{1}{2}$  hours. At the end of this time 1.3 cc. of the serum were carefully pipetted off the top of the sample. The protein

content of this fraction was 5.6 gm. per 100 cc. 5 cc. more were withdrawn off the top of the sample and discarded. The protein content of the remaining 3.5 cc. of serum, after thorough stirring, was 12.7 gm. per 100 cc.

The protein partition of the original and concentrated serum of twelve samples was determined to see whether the state of aggregation of the protein had been altered during the concentrating. The results are given in Table I. Concentrating the serum did not change the ratio of albumin to globulin, for in every case the

TABLE I  
*Protein Concentration and Albumin to Globulin Ratios in Original and Concentrated Samples on Dog Sera*

Date	Dog	Total protein		Per cent increase of protein	Albumin to globulin ratio		
		Original serum	Concentrated serum		Original serum	Concentrated serum	Difference
		gm. per 100 cc.	gm. per 100 cc.				
July 11, 1934	C	6.1	8.8	42	1.3	1.3	0.0
" 26, 1934		6.2	10.5	69	0.8	0.8	0.0
June 29, 1934	F	6.6	9.6	45	1.2	1.2	0.0
July 9, 1934		5.8	8.9	53	1.2	1.3	-0.1
" 17, 1934		6.0	9.6	60	1.2	1.3	-0.1
Oct. 5, 1934	D	6.1	8.8	44	1.4	1.5	-0.1
Feb. 28, 1935		5.8	8.9	53	1.6	1.6	0.0
July 5, 1934	Y	6.1	8.4	38	1.1	1.3	-0.2
" 13, 1934		6.4	9.5	48	1.2	1.2	0.0
" 24, 1934		5.9	9.8	66	1.2	1.0	+0.2
June 27, 1934	X	5.8	8.7	50	1.4	1.5	-0.1
July 19, 1934		5.8	13.4	130	1.2	1.3	-0.1

ratios for the original and the concentrated serum agree within 0.2 unit. In one sample the serum was concentrated from 5.8 to 13.4 gm. per 100 cc., with a change in the albumin to globulin ratio of only 0.1 unit.

*Protein Determinations*—The protein values are calculated from the difference between the total nitrogen and the non-protein nitrogen by multiplying by the factor 6.25. The samples were digested in 100 cc. Kjeldahl flasks with 2 cc. of a sulfuric acid-selenium oxychloride mixture (1 cc. of selenium oxychloride added

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to a mixture of 250 cc. of concentrated  $\text{H}_2\text{SO}_4$  and 250 cc. of saturated  $\text{K}_2\text{SO}_4$  solution). The distillation of the ammonia was carried out in the steam distillation apparatus of Goebel, illustrated by Peters and Van Slyke ((1932) p. 531). The ammonia was collected in 0.01 N hydrochloric acid and the excess acid titrated with 0.01 N sodium hydroxide, with methyl red as an indicator. In our experience selenium as a catalyst is preferable to copper or persulfate. The non-protein nitrogen of serum was determined on a trichloroacetic acid filtrate. On the ultrafiltrate total nitrogen was determined and assumed to be non-protein nitrogen, since no protein could be detected and since the nitrogen values always approximated the non-protein nitrogen of the serum. In the fractionation of the protein, the Howe (1921) precipitation procedure with 22 per cent  $\text{Na}_2\text{SO}_4$  solution was used. The precipitations and filtrations were made in a 38° constant temperature room.

*Electrometric pH Determination and Equilibration of Serum*—The electrometric pH determinations were made in Clark-Cullen and Simms bubbling hydrogen electrode vessels. All determinations were made at 38° and all the values are based on assigning to the reference solution, 0.1 N hydrochloric acid, a pH of 1.080.

The Simms bubbling hydrogen electrode vessel (1923), as used by us (1934) for  $\text{pK}'$  determinations, also furnishes a convenient method for equilibration of serum with carbon dioxide-hydrogen gas mixtures. The technique has been described in detail previously (see Robinson, Price, and Cullen (1934) p. 13). The carbon dioxide-hydrogen gas mixtures were furnished in high pressure cylinders by the Ohio Chemical Company. The percentages of  $\text{CO}_2$  in the two tanks used in these experiments were 5.19 and 5.89, respectively. The contents of these tanks were analyzed at intervals during the experimental period and found to remain uniform in composition.

When gas is bubbled through serum in the Simms electrode vessel, octyl alcohol must be used to prevent excessive foaming. The octyl alcohol (Eastman) has practically no influence on the electrometric pH determination. However, after bubbling gas through serum containing a drop of octyl alcohol for a few minutes, the serum becomes quite turbid. To determine whether this turbidity might have such an influence on the dye color that  $C$

corrections could not be comparable to those that would be obtained on the original serum, it was necessary to compare the *C* corrections determined on the equilibrated serum in the Simms vessel with the value obtained on the same serum that had no octyl alcohol added to it or no gas bubbled through it. Since the pH determination in the Clark hydrogen electrode vessel has been the basis of most previous work on *C* correction and since serum rocked in that vessel does not become markedly turbid, experi-

TABLE II

*Comparison of C Corrections of Dog Sera Obtained by Equilibration in Simms Bubbling Hydrogen Electrode with CO<sub>2</sub>-H<sub>2</sub> Mixture with Those Obtained by Refill Technique in Clark Hydrogen Electrode*

Dog	Simms electrode			Clark electrode			<i>C</i> difference, Clark minus Simms
	pH <sub>est</sub> <sup>o</sup>	[pH] <sub>20</sub> <sup>o</sup>	<i>C</i>	pH <sub>est</sub> <sup>o</sup>	[pH] <sub>20</sub> <sup>o</sup>	<i>C</i>	
E	7.45	7.78	0.33	7.40	7.72	0.32	-0.01
D	7.39	7.70	0.31	7.40	7.72	0.32	+0.01
A	7.34	7.71	0.37	7.39	7.77	0.38	+0.01
D	7.35	7.64	0.29	7.37	7.67	0.30	+0.01
B	7.37	7.68	0.31	7.40	7.71	0.31	0.00
F	7.37	7.67	0.30	7.36	7.64	0.28	-0.02
X	7.39	7.68	0.29	7.38	7.72	0.34	+0.05
AB	7.30	7.63	0.33	7.36	7.68	0.32	-0.01
X	7.45	7.77	0.32	7.41	7.76	0.35	+0.03
Y	7.33	7.68	0.35	7.35	7.72	0.37	+0.02
D	7.37	7.68	0.31	7.43	7.75	0.32	+0.01
X	7.39	7.68	0.29	7.38	7.70	0.32	+0.03
F	7.31	7.67	0.36	7.32	7.67	0.35	-0.01

ments were planned to compare *C* corrections made with these two vessels.

The experiments were carried out as follows: Serum was obtained with the necessary precautions to prevent loss of CO<sub>2</sub> and a colorimetric pH determination was made and used in calculating the CO<sub>2</sub> tension of the serum. CO<sub>2</sub>-H<sub>2</sub> mixtures were then prepared at approximately that CO<sub>2</sub> tension. This CO<sub>2</sub>-H<sub>2</sub> mixture was then used for the pH determinations with the Clark-Cullen vessel and for about one-third of the determinations in the Simms vessel (reported in Table II), those where pH<sub>est</sub> values of



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the two electrodes agree within 0.02 pH. In the remainder of the values reported in Table II, the tank  $\text{CO}_2\text{-H}_2$  mixtures were used for the Simms electrode. For the determination in the Clark vessel, the vessel was first washed with the  $\text{CO}_2\text{-H}_2$  gas mixture and then the serum allowed to displace some of the gas mixture. The Hasselbalch refill technique was used until, on successive renewals of serum, with the same  $\text{CO}_2\text{-H}_2$  gas bubble, the E.M.F. readings were constant. The colorimetric pH determinations used for the  $C$  values were then made on serum removed from the vessels without loss of  $\text{CO}_2$  with a pipette, from the Simms vessel by removal of the electrode, and from the Clark-Cullen vessel by removal of the thermometer.

The results obtained on thirteen parallel determinations with the two equilibration techniques are shown in Table II. It is apparent that the agreement obtained warranted the use of dog serum equilibrated in the Simms vessel for this study. Although this statement is true for dog serum, experiments not reported here have shown that it does not always hold for serum of other species, but that equilibration sometimes changes the characteristics of the serum.

*Colorimetric pH Determinations*—The phosphate buffer mixtures at 0.05 pH intervals were made from M/7.5 stock solutions of disodium hydrogen phosphate,  $\text{Na}_2\text{HPO}_4$ , and potassium acid phosphate,  $\text{KH}_2\text{PO}_4$  (Merck's Sørensen's salts). In making a standard the M/7.5 buffer mixture was mixed with an equal volume of a 0.0016 per cent phenol red solution. This diluted dye solution was prepared by diluting 8 cc. of the stock 0.04 per cent phenol red solution to volume in a 200 cc. volumetric flask with freshly boiled redistilled water immediately before making up a set of standards. The 0.04 per cent phenol red solution is obtained by diluting a 0.1 per cent stock solution that is prepared as recommended by Clark (1928). A new set of color standards was made up every week. In these experiments the pH values of the color standards were checked daily both with a glass electrode and spectrophotometrically (reading at wave-length  $560\text{ m}\mu$ ). The color is stable for at least a week. The phosphate buffer solutions must be protected from the  $\text{CO}_2$  of the air, for they may take up enough  $\text{CO}_2$  to alter the pH by 0.02 unit. This error was encountered when attempting to compare the electro-

metric pH value obtained in the bubbling hydrogen electrode with those obtained by the glass electrode. On old solutions the glass electrode values were more acid than those of the Simms electrode. However, if CO<sub>2</sub>-free air or hydrogen was bubbled through the solution, the glass electrode reading agreed with the Simms electrode. That is, the Simms electrode was determining the pH of a CO<sub>2</sub>-free solution.

The saline-dye mixture was made by measuring with a special Van Slyke-Ostwald pipette 2.1 cc. of a 0.04 per cent phenol red solution into a 100 cc. volumetric flask and diluting to the mark with a 0.919 per cent sodium chloride solution. The final dye concentration in the saline-dye mixture after it has been diluted 1:20 with serum is 0.0008 per cent. In order to free the saline-dye mixture of CO<sub>2</sub> and to adjust the pH to about 7.5, CO<sub>2</sub>-free air is drawn through the solution. Air was freed of CO<sub>2</sub> in a train consisting of a Milligan gas-washing bottle containing a saturated solution of sodium hydroxide, followed by a Milligan bottle containing saline and phenol red. The latter bottle serves to saturate the air with water vapor and the presence of the dye allows detection of alkali that might be carried over in the spray. The CO<sub>2</sub>-free air passes through the saline-dye mixture in small bubbles. 10 minutes usually suffice for removal of all the CO<sub>2</sub> and to bring the solution to the proper reaction. A fresh saline-dye mixture was prepared each morning.

Uniform test-tubes of clear "government ampule" glass, 20 mm. in internal diameter, were used as containers for the phosphate buffer standards and the saline-dye-serum samples. Comparisons were made in a Walpole comparator against a daylight lamp. The lamp was constructed from a Palo lamp dome and the housing of the Bausch and Lomb colorimeter lamp. Turbidity and color in the sample were compensated for in the usual manner. It has been our experience that a more uniform tone of color for comparison is obtained when the saline-dye-serum tube and the saline-serum control tube are placed in the rear row (nearest to the source of light) and the phosphate standards and water tubes in the front row of the comparator block.

We emphasize these details of technique because it is our conviction that some of the variations in colorimetric pH values reported from different laboratories are due to reading colors

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under definitely different conditions, including light source, focus, types of color filters or colored bulbs, diameters of the tubes, concentrations of dyes, and in fact all details which affect the *virage* of the solution.

### *Results*

Dog blood was obtained, without anesthesia, from the heart through a 4 inch, 16 gage lumbar puncture needle. The blood was drawn directly under oil into a 50 cc. Pyrex centrifuge tube. The tube was centrifuged immediately without further procedure to minimize loss of  $\text{CO}_2$ . A sample was placed in the ultrafiltration apparatus and filtered under a pressure of 150 pounds per sq. inch. In order to speed up the filtration, the pressure was released at 2 hour intervals, the top *D* was removed, and the contents stirred carefully with a glass rod, care being taken not to injure the membrane. The ultrafiltrate was collected in a clean dry test-tube. The average time to obtain the concentrated serum was about 6 hours.

The original serum was diluted with ultrafiltrate to obtain protein concentrations lower than those of the original serum. Intermediate concentration points between the original and concentrated serum were obtained by mixing. The original serum, the concentrated, the ultrafiltrate, and the mixtures were equilibrated in the Simms electrode vessel with the same  $\text{CO}_2\text{-H}_2$  gas mixture. After constant E.M.F. readings were obtained, the hydrogen electrode was removed from the Simms vessel and 0.5 cc. of the solution was removed by a Van Slyke-Ostwald pipette. This sample was introduced into a tube containing 10 cc. of the adjusted, oil-covered saline-dye mixture. For a control tube another 0.5 cc. of the sample was introduced into 10 cc. of saline solution. The tubes were removed at once to a  $20^\circ$  constant temperature room and the color readings were made at  $20^\circ$  in about  $\frac{1}{2}$  hour.

The results for twenty-eight samples of serum from eight normal dogs are given in Table III.

The pH and  $\text{CO}_2$  content values were practically constant for the samples from a single specimen of serum. The pH values never varied more than 0.04 pH and the  $\text{CO}_2$  contents were generally within 1 volume per cent. There was a tendency for the pH of

TABLE III

Variation of C Correction with Protein Concentration on Dog Serum Equilibrated at 38° with CO<sub>2</sub>-H<sub>2</sub> Mixture (Per Cent CO<sub>2</sub> = 5.19 or 5.89) in Simms Electrode Vessel

Dog	Date	Sample	pH <sub>m</sub> <sup>a</sup>	CO <sub>2</sub>	pCO <sub>2</sub>	Non-protein N	Protein	C
				vol. per cent	mm.	mg. per cent	per cent	
Y	July 5, 1934	Original	7.30	47.0	40.7	32	6.1	0.29
		Concentrated	7.29	46.9	40.6	35	8.4	0.33
		Original	7.28	44.9	40.5	32	6.4	0.30
	" 13	Concentrated	7.28	44.7	40.4	33	9.5	0.33
		Mixture	7.26	44.9	40.4		3.2*	0.25
		Original	7.23	40.3	40.7	21	5.9	0.27
	" 24	Concentrated	7.23	40.9	40.6	22	9.8	0.31
		Mixture	7.21	40.0	40.7		2.95	0.20
		Original	7.35	46.7	36.3	25	6.0	0.32
	Oct. 1	Concentrated	7.35	46.4	36.2	27	8.1	0.33
		Mixture	7.35	47.7	36.3		3.0	0.23
		Original	7.33	49.5	40.9	33	5.8	0.30
	" 17	Concentrated	7.33	49.2	40.9	34	8.7	0.34
		Original	7.34	51.3	40.5	29	5.8	0.33
	" 24	Concentrated	7.34	50.3	40.4	30	11.8	0.36
		Mixture	7.34	51.0	40.5		4.35	0.28
		"	7.34	51.4	40.5		2.9	0.25
	Nov. 8	Original	7.34	45.8	35.9	31	6.4	0.37
		Concentrated	7.33	45.2	36.2	35	11.6	0.40
		Ultrafiltrate	7.31		35.9	30		0.22
	Dec. 10	Original	7.39	50.8	35.8	30	6.1	0.28
		Concentrated	7.39	50.8	35.8	31	8.5	0.33
		Mixture	7.39	50.9	35.8		4.6	0.25
	" "	"	7.38	51.1	35.8		3.0	0.23
		Original	7.44		36.0	25	5.8	0.32
		Concentrated	7.44		36.0	26	8.1	0.37
F	June 29, 1934	Original	7.29	45.9	40.8	33	6.6	0.28
		Concentrated	7.29	45.9	40.7	34	9.6	0.32
		Ultrafiltrate	7.25	45.6	40.7	32		0.22
	July 9	Original	7.28	45.1	41.0	27	5.8	0.30
		Concentrated	7.28	44.1	41.0	28	8.9	0.34
		Ultrafiltrate	7.25	44.6	41.0			0.19
	" 17	Original	7.25	42.3	40.7	29	6.0	0.32
		Concentrated	7.25	41.9	40.7	32	9.6	0.39

<sup>a</sup> Protein values for mixtures calculated.

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TABLE III—Continued

Dog	Date	Sample	pH <sub>est</sub> <sup>a</sup>	CO <sub>2</sub>	pCO <sub>2</sub>	Non-protein N	Protein	C
				vol. per cent	mm.	mg. per cent	per cent	
F— Con- tin- ued	July 27	Original	7.29	46.3	40.6	34	7.3	0.33
		Concentrated	7.29	46.3	40.5	35	9.0	0.37
		Ultrafiltrate	7.25		40.6	33		0.18
	Nov. 15	Original	7.38	51.0	36.5	35	6.2	0.39
		Concentrated	7.36	49.4	36.3	36	11.0	0.47
		Mixture	7.36	50.1	36.3		8.6	0.44
		"	7.39	52.2	36.4		3.1	0.30
	" 26	Ultrafiltrate	7.40	54.0	36.4	34		0.16
		Original	7.28	40.5	36.0	26	6.0	0.34
		Concentrated	7.28	40.0	36.2	28	11.8	0.39
		Mixture	7.28	40.6	36.0		3.0	0.27
		Ultrafiltrate	7.26	40.0	36.1	25		0.13
X	June 27	Original	7.31	47.5	40.7	29	5.8	0.31
		Concentrated	7.31	47.3	40.7	31	8.7	0.36
		Ultrafiltrate	7.25	46.5	40.7	30		0.25
	July 19	Original	7.38	55.5	40.7	27	5.8	0.29
		Concentrated	7.38	55.6	40.7	33	13.4	0.39
	Nov. 5	Original	7.39	51.5	35.7	22	5.3	0.33
		Concentrated	7.38		35.7	22	7.5	0.37
		Ultrafiltrate	7.38	51.9	35.7	20		0.12
	Dec. 3	Original	7.44	56.8	35.8	29	5.6	0.35
		Concentrated	7.44	56.4	35.8	32	8.1	0.39
		Mixture	7.44	58.0	35.8		2.8	0.27
		Ultrafiltrate	7.45	57.6	35.8			0.15
	Jan. 22, 1935	Original	7.38	56.5	41.0	32	6.3	0.39
		Concentrated	7.36	54.2	41.0	36	10.7	0.42
		Mixture	7.37	55.0	40.8		4.8	0.36
C	July 11, 1934	Original	7.32	49.3	40.8	33	6.1	0.31
		Concentrated	7.32	49.2	40.7	34	8.8	0.35
		Mixture	7.30	48.2	40.7		3.05	0.24
D	Oct. 5	Ultrafiltrate	7.29	48.4	40.8	31		0.15
		Original	7.34	45.5	36.1	28	6.1	0.38
		Concentrated	7.33	44.5	36.1	30	8.8	0.41
		Mixture	7.34	45.5	36.1		4.6	0.33
	Feb. 28, 1935	"	7.34	45.9	36.1		3.05	0.30
		Original	7.38		36.4	32	5.8	0.40
		Concentrated	7.37		36.4	33	8.9	0.46
		Ultrafiltrate	7.37		36.4			0.20

TABLE III—*Concluded*

Dog	Place	Sample	pH <sub>est</sub> *	CO <sub>2</sub>	pCO <sub>2</sub>	Non-protein N	Protein	C
				vol. per cent	mm.	mg. per cent	per cent	
AB	Oct. 11, 1934	Original	7.36	47.5	35.9	21	5.4	0.28
		Concentrated	7.37	48.0	35.8	22	9.2	0.35
		Mixture	7.37	49.6	35.8		2.7	0.25
Z	Dec. 13	Original	7.43	56.5	35.9	28	5.0	0.34
		Concentrated	7.37	50.1	36.3	30	9.9	0.45
		Mixture	7.43	55.1	36.0		3.75	0.30
	" 17	"	7.43	57.0	36.0		2.5	0.27
		Ultrafiltrate	7.42	57.1	36.0	26		0.15
		Original	7.44	57.8	36.1	30	4.6	0.38
AA	" 19	Concentrated	7.44	57.2	36.2	30	7.0	0.42
		Ultrafiltrate	7.43		36.1	29		0.21
		Original	7.37	48.8	35.0	36	5.0	0.39
		Concentrated	7.36	47.3	35.3	38	7.1	0.41
		Ultrafiltrate	7.35		35.1	31		0.20

the ultrafiltrate on equilibration to be about 0.03 pH more acid than the pH values of the original and concentrated sera which were usually within 0.01 pH of each other. As we did not attempt to control the partial pressure of CO<sub>2</sub> in the gas phase over the serum in the ultrafiltration apparatus, the pH of the unequilibrated ultrafiltrate was very alkaline (pH about 8.2).

The percentage of diffusible calcium in serum has been shown by a number of workers to be dependent on the reaction of the serum. The calcium was determined on a few specimens and the values agree approximately with those in the literature. Table IV gives representative values of the calcium concentration in original, concentrated serum, and ultrafiltrate. The calcium concentration in the concentrated serum increases in direct relation to the protein concentration. Chloride determinations were made on a number of samples, and in every case the chloride concentration was greater in the ultrafiltrate, and less in the concentrated serum, than in the original serum (see Table V). The successive portions of the ultrafiltrate vary slightly in composition.

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Analyses of the chloride in successive fractions of the ultrafiltrate are given for one sample of serum in Table VI. The difference in chloride between the first and last fractions was only 2.8 mm per liter.

TABLE IV  
*Calcium Determinations in Serum (in Mg. per 100 Cc.)*

Date, 1934.....	Dog Y July 24	Dog F July 17	Dog X July 19
Original serum.....	10.0	10.2	11.0
Concentrated.....	13.0	12.6	
Ultrafiltrate.....	4.4	5.1	5.2

TABLE V  
*Chloride Determinations (in mM per Liter) on Original Serum, Concentrated Serum, and Ultrafiltrate*

Dog..... Date, 1934.....	Y July 5	Y July 13	Y July 24	Y Oct. 17	F July 9	C July 11	AB July 15
Original serum.....	108.1	114.3	112.0	103.9	105.3	114.4	109.2
Concentrated.....	99.7	106.2		98.2	98.6	109.1	92.4
Ultrafiltrate.....	119.6	126.3	124.1	114.1	116.7	125.4	119.4

TABLE VI  
*Chloride Concentration in Ultrafiltrate Fractions*  
Dog C; July 26, 1934.

Fraction No.	Volume of fraction	Cl
	cc.	mM per l.
1	1.3	125.5
2	1.3	125.8
3	1.9	126.3
4	2.5	128.3

As sodium chloride is the principal electrolyte in serum, it was important to know whether such changes in chloride concentration, as are shown in Table V, would affect the *C* correction. The results of experiments, in which dry sodium chloride was dissolved in serum showed that this change in chloride concentration between the ultrafiltrate and the concentrated serum had no influence on the *C* correction. In the first experiment recorded in

Table VII, changes up to 20 mm per liter of NaCl did not change *C* corrections beyond the limits of experimental error. The difference in the chloride concentration between the original serum and the concentrated serum was never greater than 17 mm per liter and the difference between the concentrated and ultrafiltrate was never greater than 27 mm per liter. Therefore, the change in sodium chloride concentration in the various samples is not a factor that influences the variation of the *C* correction. The second experiment in Table VI shows the effects that much larger quantities of salt have on the *C* correction. Only with increases

TABLE VII

*Effect of NaCl on C Correction of Dog Serum Equilibrated at 38° in Simms Vessel*

Dog and date (1935)	Sample	[Cl]	C	Protein	Non-protein N	pH <sub>est</sub>	[CO <sub>2</sub> ]	pCO <sub>2</sub>
		mm per l.		gm. per 100 cc.	mg. per 100 cc.		mm per l.	mm.
D	A = original	120.6	0.38	6.2	36	7.342	23.4	40.2
Feb. 14	" + 10 mm per liter NaCl	130.8	0.38	6.2		7.337		40.2
	" + 20 " " " "	140.4	0.39	6.2	35	7.336	23.5	40.2
Y	" = original	117.8	0.35	6.4	31	7.392	26.1	40.8
Feb. 11	" + 100 mm per liter NaCl	218.1	0.36	6.4	31	7.360	26.1	40.7
	" + 200 " " " "	318.4	0.39	6.4	31	7.334	26.1	40.7
	" + 300 " " " "	418.7	0.41	6.35	32	7.313	26.1	40.7

in salt concentration of 200 to 300 mm per liter is the *C* correction influenced.

The differences in concentration of non-protein nitrogen in the various samples must have a negligible effect on the color correction. On an average the ultrafiltrate contained 1 to 2 mg. less of non-protein nitrogen and the concentrated serum contained a few mg. more than the original serum. Although always determined, the ultrafiltrate nitrogen values are given in Table III only when other analyses are reported.

Since in every experiment the *C* correction increases with the protein concentration, and since the other constituents such as sodium, potassium, and total electrolytes were not significantly



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changed in the different samples, it must be concluded that the  $C$  corrections are varying, either with the protein concentration, or with some constituent that is not ultrafiltrable and is associated with the protein. If the values of the  $C$  correction are plotted against the logarithm of the protein concentration (in gm. per 100 cc.), the points of the samples from a single serum fall on a straight line and the values of the slopes ( $C/(\log \text{ protein})$ ) of these straight lines in our twenty-eight experiments are between a maximum of 0.350 and a minimum of 0.170. Fig. 3 shows a few of these experiments. The mean value of  $C/(\log \text{ protein})$  is 0.243,

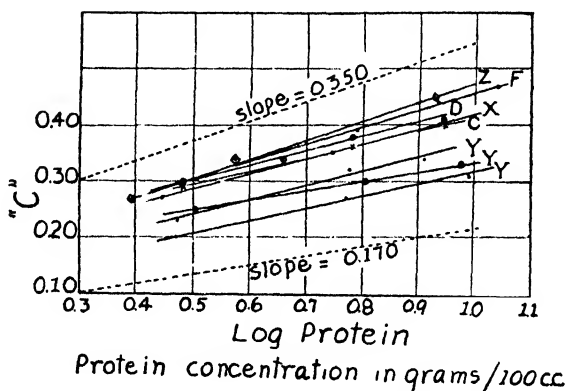


FIG. 3. The relation of  $C$  correction to protein concentration (plotted logarithmically) of a representative group of experiments. The dotted lines represent the maximum and minimum slopes obtained in the entire series. The capital letters represent the individual dogs referred to in the tables.

and for thirteen experiments in which there are three or more points the values of the slopes are distributed between 0.170 and 0.299.

Although in every case the  $C$  correction has varied directly with the protein, the protein concentration of a serum does not determine the exact value of the  $C$  correction. At various times sera from the same dog have shown marked variation in the  $C$  correction, although the protein concentrations have remained approximately constant. Such a case is shown for Dog F on July 17 and November 15 (Table III), where the total protein concentration is 6.0 and 6.2 gm. per 100 cc., respectively. In such a case, if

protein were the only factor, the  $C$  corrections should be the same. However, values of 0.32 and 0.39 were obtained.

It is, therefore, necessary to conclude that, although there is a definite relationship between the protein concentration and the  $C$  value, there are other, as yet undetermined, factors which have a greater influence on the value of the  $C$  correction of a sample of serum than changes in protein concentration alone.

Although the experiments reported here were all made on dog sera, and it is sometimes dangerous to translate the results of experiments from one species to another, there are several considerations which indicate that the relationship between  $C$  and protein concentration is the same for human serum as it is for dog serum. In the first place the average  $C$  correction of dog sera, 0.34, is not far from the average for normal human sera. In the second place, the magnitude of the change in the  $C$  correction with change in protein concentration observed by Myers, Muntwyler, Binns, and Danielson on diluting pooled human serum with a mixture of salts that kept  $\text{CO}_2$ , chloride, phosphate, and pH approximately constant is of the same order of magnitude as that observed in our experiments with dogs reported above.

The third fact, most important for this argument, is that if a straight line is fitted, by the method of least squares, to the values for human serum reported in Fig. 1, the slope  $C/(\log \text{protein})$  of 0.283 is close to the average value of 0.243 for the equilibration experiments on dog sera. Therefore, the change in  $C$  between an average normal human serum, with a protein concentration of 6.5 gm. per 100 cc., and an average nephrotic human serum, with a protein concentration of 3.5 gm. per 100 cc., is approximately the same as the change in  $C$  for a change in protein concentration of 3 gm. per 100 cc. in dog serum.

It seems evident, therefore, that the lowered  $C$  correction in the nephrotic patients was due to the low protein concentration.

#### SUMMARY

Dog serum was concentrated by ultrafiltration through a cellophane membrane (du Pont No. 300) at 150 pounds per sq. inch. Concentrating serum in this manner does not change the albumin to globulin ratio. Mixtures were made of the original and concentrated serum and of the original serum and its ultrafiltrate.

The  $C$  correction (colorimetric pH of serum diluted 1:20 in

saline-phenol red solution minus the pH of the undiluted serum at 38° determined by the hydrogen electrode) on these mixtures equilibrated at 38° with the same CO<sub>2</sub>-H<sub>2</sub> mixture in the Simms bubbling hydrogen electrode vessel was found to vary directly with the logarithm of the protein concentration (in gm. per 100 cc.). The average value of  $C/(\log \text{ protein})$  for twenty-eight samples of serum from eight normal dogs is 0.243; the maximum and minimum values are 0.350 and 0.170, respectively.

The change in  $C$  correction with change of protein is about the same for equilibrated dog and human sera. This explains the difference in  $C$  corrections observed between normal human serum and nephrotic human serum.

The experiments also show that in dog sera other factors must be present at various times, that have a greater influence on the absolute magnitude of the  $C$  correction than does change in protein concentration.

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# COMPOUNDS OF THIOL ACIDS WITH ALDEHYDES

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It has been shown (1) that both methylglyoxal and phenylglyoxal combine with thiol acids. Two types of compound were described. The first is a simple addition compound composed of 1 molecule of glyoxal and 1 of thiol, while the second is a condensation product formed by eliminating a molecule of water between a molecule of glyoxal and 1 of thiol. The second type is given only by cysteine and by thiourea.

So far it has been impossible to determine the structure of these compounds, as they appear to dissociate into their components quite readily. For example, on treatment of the phenylglyoxal compounds with phenylhydrazine the osazone of phenylglyoxal is formed. However, it has now been found that the interaction of thiols and carbonyl compounds is much more general than was at first suspected and compounds of both the types mentioned above have been prepared with simple aldehydes. It seems better, therefore, to begin the study of the behavior of these compounds with the simplest possible combinations of thiol and carbonyl.

Although thioglycolic acid is the simplest thiol acid, it was to be expected that if addition of thiols to simple aldehydes occurs quite generally, thioglycolic acid anilide would give such compounds in crystalline form more readily. This turned out to be the case and addition compounds were easily obtained with formaldehyde, butyric aldehyde, and chloral. The only simple imaginable structure for these compounds is



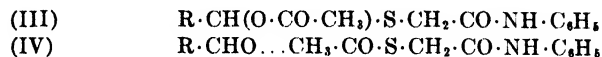
These compounds are made at room temperature with neutral solvents, and are stable enough to be recrystallized repeatedly.

One of their most striking properties is the ease with which they appear to dissociate into their components, aldehyde and thiol, as illustrated by the following behavior. When suspended in aqueous bicarbonate and treated with sodium nitroprusside, a color is produced, although it is much weaker than that formed in a similar suspension of thioglycolic acid anilide itself. An alcoholic solution of any one of these three compounds can be titrated with alcoholic iodine just as readily and as sharply as thioglycolic acid anilide. In each case an atom of iodine is consumed for each molecule of compound and dithiodiglycolic acid dianilide is produced. A further example, which can be interpreted on the supposition that compounds of the form (I) dissociate in solution, is the following. If a solution of the phenylglyoxal-thioglycolic acid compound in alcohol be treated at room temperature with thioglycolic acid anilide, an exchange occurs and the much less soluble phenylglyoxal-thioglycolic acid anilide compound is formed and crystallizes out almost quantitatively.

In view of these properties some doubt might be cast on the structure (I), which represents a thio ether and as such would be expected to be more stable. Because these compounds dissociate so readily, it could be argued that they are some sort of loose molecular compound to which no particular valence formula can be assigned, so that they must be written as



In favor of the formulation (I) are the following facts. The addition compounds acetylate readily, giving compounds (III) or (IV) derived from (I) or (II) respectively.

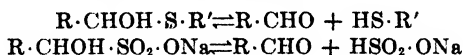


If thioglycolic acid anilide itself be acetylated, however, the product gives no addition compound with aldehydes, as would be expected if (IV) represented the acetylated addition compounds. Thus the structures (I) and (III) are to be preferred. These compounds would be called 1-hydroxyalkylthioglycolic acid anilides.

The acetylated compounds (III) also consume iodine, but do so only very slowly, so that it is impossible to titrate them. When

suspended in sodium bicarbonate solution or even weak ammonia solution, they give no nitroprusside test at all. Thus acetylation of the hydroxyl group in (I) seems to stabilize the compounds so that they do not dissociate so readily.

The peculiar instability conferred on a thio ether, when the carbon adjacent to the sulfur carries a hydroxyl group, is like the instability of sulfonic acids under similar conditions. In each case the compounds dissociate readily.

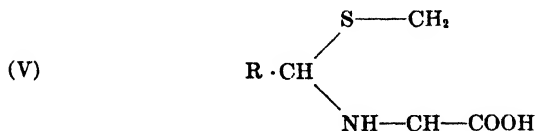


The sulfonic acid structure of the aldehyde-bisulfite compounds has been quite thoroughly discussed in the work of Raschig and Prahl (2) and further evidence has more recently been presented by Lauer and Langkammerer (3).

Of the ketones tried, acetone, acetophenone, diacetyl, and benzil give no evidence of combination with thioglycolic acid anilide but pyruvic acid does give an addition compound. This compound consumes iodine just as the aldehyde compounds already described, dithiodiglycolic acid dianilide being produced. Dissolved in sodium bicarbonate the compound gives a strong nitroprusside reaction.

Besides the carbonyl compounds already mentioned, quinone and isatin give addition compounds with thioglycolic acid anilide. The quinone compound is of particular interest, as it may have some relation to the colored compounds produced in Sullivan's cysteine reaction or in that of Baudisch.

Cysteine has been found to give with simple aldehydes condensation products analogous to its compound with phenylglyoxal previously described. Such compounds have now been prepared with formaldehyde,<sup>1</sup> butyric aldehyde, chloral, benzaldehyde, and furfural. The most probable structure for these compounds is



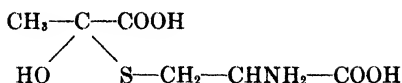
<sup>1</sup> This compound has been independently discovered by Dr. H. T. Clarke in whose laboratory work is now being carried out on its structure and properties.

In support of this formulation are the properties of these compounds. The carboxyl group seems to be free, since the compounds have acid properties, displacing carbon dioxide from bicarbonate solution and even the most insoluble dissolving in water on the addition of an excess of sodium acetate. All of these compounds, except that with chloral, give negative nitroprusside tests in bicarbonate solution. In weak ammonia the benzaldehyde and furfural as well as the chloral compounds give positive tests and the compound with butyric aldehyde gives a very faint test, while the phenylglyoxal and formaldehyde compounds give no tests at this alkalinity. It thus appears that the thiol group takes part in the compound formation and that these compounds dissociate to different extents, depending on the nature of the aldehyde and on the alkalinity. An interesting experiment indicating that dissociation probably occurs to a slight extent even when a nitroprusside test gives a negative result is as follows: If the compound formed from cysteine and benzaldehyde is neutralized with sodium bicarbonate and is treated with sodium iodoacetate, there soon appears a precipitate of benzaldehyde and after a few hours it is possible to isolate from the solution the mixed sulfide,  $\text{HOOC} \cdot \text{CH}_2 \cdot \text{S} \cdot \text{CH}_2 \cdot \text{CHNH}_2 \cdot \text{COOH}$ , which has already been shown to be formed by the action of iodoacetic acid on cysteine (4). Further evidence that this compound dissociates in solution is found in the ready formation of benzaldehyde phenylhydrazone on addition of phenylhydrazine.

These cysteine-aldehyde compounds absorb iodine just as readily as cysteine, an atom of iodine being absorbed for each molecule of compound and cystine being produced. Two of them, the phenylglyoxal and the benzaldehyde compounds, have been acetylated. These acetylated compounds just like the acetylated aldehyde-thioglycolic acid anilide compounds only very slowly absorb iodine.

All of the cysteine-aldehyde compounds mentioned so far are formed by condensation with loss of a molecule of water. An exception to this is the cysteine-pyruvic acid compound which is formed by simple addition of the two acids. This compound also gives a negative nitroprusside test in sodium bicarbonate as well as weak ammonia solution. Like the other addition compounds its structure is probably

(VI)



On treatment with acetic anhydride it gives a diacetate, supporting the formulation (VI). This acetate, like those previously described, gives no color with nitroprusside even in weak ammonia and absorbs iodine only extremely slowly, while (VI) can be titrated with iodine, yielding cystine.

These aldehyde-thiol compounds, because of their reversible formation and dissociation in neutral aqueous solution at room temperatures, are of some biochemical interest. For example, a thiol like cysteine can be masked in the presence of aldehydes so that, although it can react with reagents such as iodoacetic acid, its presence might not be detected by a nitroprusside test. The effect can also be thought of as a "buffering" of thiol concentration.

#### EXPERIMENTAL

Thioglycolic acid anilide is prepared by the method of Jäger (5) as modified by Beckurts and Frerichs (6), and has a melting point of 113–114°. Its compounds with aldehydes are all prepared similarly. About 18 gm. of thioglycolic acid anilide are suspended in 50 cc. of alcohol at room temperature. Most of the anilide remains undissolved. An excess of the aldehyde is then added, 15 cc. of an aqueous 40 per cent formaldehyde solution, 12 cc. of butyric aldehyde, or 25 gm. of chloral hydrate. After the aldehyde has been added, the mixture is stirred and in 10 to 20 minutes a clear solution results. This is allowed to stand for 2 hours, then about 100 cc. of water are added. The product separates as an oil, which, after a sufficient amount of scratching, crystallizes. The mixture is set on ice for a few hours. The product is then filtered off, sucked dry, dissolved in 100 to 150 cc. of alcohol, and 2 volumes of water are added to the clear solution which is then set on ice. The crystals are filtered with suction and dried *in vacuo*. The yield varies from 12 gm. for the formaldehyde compound to 20 gm. for the chloral compound.  $\text{CH}_2\text{O} \cdot \text{C}_6\text{H}_5\text{ONS}$ , m.p. 91–92°, iodine value 0.95; calculated, S 16.24, N 7.11; found, S 16.16, N 7.08.  $\text{C}_3\text{H}_7\text{CHO} \cdot \text{C}_6\text{H}_5\text{ONS}$ , m.p. 73–75°, iodine value 1.07; calculated, S 13.39, N 5.86; found, S 13.28, N 5.74.  $\text{CCl}_3\text{-}$



$\text{CHO} \cdot \text{C}_8\text{H}_9\text{ONS}$ , m.p. 111–112°, iodine value 1.00; calculated, S 10.19, N 4.46, Cl 33.92; found, S 10.32, N 4.66, Cl 34.32.

The pyruvic acid compound can be prepared in the same way. It often crystallizes directly from the alcohol but the addition of water hastens the separation and increases the yield.  $\text{C}_8\text{H}_4\text{O}_8 \cdot \text{C}_8\text{H}_9\text{ONS}$ , m.p. 90–92°, iodine value 1.3; calculated, S 12.55, N 5.49; found, S 12.50, N 5.35.

These compounds can be titrated with 0.1 N iodine in alcohol. A weighed sample is dissolved in 15 cc. of alcohol to which are added 2 cc. of aqueous 4 M potassium acetate. The end-points are quite sharp. The iodine value given with each compound above is the ratio of the number of equivalents of iodine consumed to the number of moles of compound weighed out. The corresponding value for thioglycolic acid anilide itself is 0.97. In each case the titration mixture is set on ice and the dithiodiglycolic acid dianilide separates, recognizable by its crystal form, its melting point of 160°, and in a few cases by analysis.

The formaldehyde and the chloral compounds have been acetylated by treating, at room temperature, with 5 parts of a mixture of equal weights of pyridine and acetic anhydride. After standing 6 to 8 hours the clear solutions are poured into 10 volumes of water and the oil which separates crystallizes easily. The product is filtered off and recrystallized from a small volume of hot alcohol.

The acetoxymethylthioglycolic acid anilide separates as flat bars, m.p. 91–92°, and with the following analysis.  $\text{C}_{11}\text{H}_{18}\text{O}_3\text{NS}$ , calculated, S 13.39, N 5.86; found, S 13.35, N 5.96.

1-Acetoxy-2,2,2-trichloroethylthioglycolic acid anilide crystallizes as rhombic plates with a melting point of 133–134°.  $\text{C}_{12}\text{H}_{12}\text{O}_3\text{NSCl}_3$ , calculated, S 8.99, N 3.93, Cl 29.93; found, S 8.81, N 4.10, Cl 29.57.

Thioglycolic acid anilide can be acetylated by the same procedure and gives, on recrystallization from hot alcohol, large rectangular plates with a melting point of 91°.  $\text{C}_{10}\text{H}_{11}\text{O}_2\text{NS}$ , calculated, S 15.31, N 6.70; found, S 15.19, N 6.57.

Acetothioglycolic acid anilide, when subjected to the action of formaldehyde or of chloral in alcoholic solution at room temperature, is unaffected and can be recovered unchanged.

All of these acetylated compounds absorb iodine, but do so so slowly that hours are required, whereas the aldehyde addition

compounds (I) can be titrated to a sharp end-point in a few minutes.

The compound of quinone and thioglycolic acid anilide is prepared by mixing clear solutions of 2 gm. of the former in 100 cc. of alcohol and 2 gm. of the latter in 50 cc. of alcohol. The solution immediately turns red and bright red crystalline plates separate in a few minutes. The product is so insoluble in all the usual solvents that it has not been possible to recrystallize it. It is merely filtered and washed with a large volume of alcohol, m.p. 165–166°.  $C_8H_4O_2 \cdot C_8H_9ONS$ , calculated, S 11.63, N 5.09; found, S 11.72, N 5.12.

The compound with isatin is prepared by adding 2.8 gm. of isatin to a solution of 3 gm. of thioglycolic acid anilide in 100 cc. of alcohol. The isatin slowly dissolves on stirring. After standing a few hours 200 cc. of water are added in portions at intervals. A crystalline product consisting of fine, sharp needles separates. This may be recrystallized by dissolving in alcohol and reprecipitating with water. When dried the product is pale yellow, m.p. 101°.  $C_8H_6O_2N \cdot C_8H_9ONS$ , calculated, S 10.19, N 8.92; found S 10.13, N 8.66.

To show the interaction of thioglycolic acid anilide with the thioglycolic acid compound of phenylglyoxal, 0.8 gm. of the former and 1.1 gm. of the latter are dissolved in 20 cc. of alcohol. After a short time a crystalline product separates and the mixture is set on ice. The product is filtered off and recrystallized from 50 cc. of hot alcohol, m.p. 148°.  $C_8H_6O_2 \cdot C_8H_9ONS$ , calculated S, 10.63, N, 4.65; found, S, 10.68, N, 4.73.

The cysteine-aldehyde compounds are all prepared similarly, the only difference being in the amount of water or alcohol used as solvent. 10 gm. of cysteine hydrochloride and 6 gm. of potassium acetate are dissolved in the following amounts of solvents and the aldehyde indicated is added: 50 cc. of water + 10 cc. of 40 per cent formaldehyde; 25 cc. of water + 10 gm. of chloral hydrate; 100 cc. of 50 per cent alcohol + 6 cc. of butyric aldehyde; 175 cc. of 50 per cent alcohol + 7 cc. of benzaldehyde; or 200 cc. of 50 per cent alcohol + 6 cc. of furfural. In each case a clear solution results on mixing, but a crystalline product begins to separate in 10 to 30 minutes. After setting on ice for some hours the products are filtered off and recrystallized as follows: The formalde-

hyde compound is dissolved in 50 cc. of water by addition of  $\text{NaHCO}_3$  and the free acid is reprecipitated by addition of acetic acid. Long rectangular needles melt at  $195^\circ$  with decomposition.  $\text{C}_4\text{H}_7\text{O}_2\text{NS}$ , calculated S 24.05, N 10.52; found, S 23.93, N 10.47. The chloral compound is twice recrystallized from 20 cc. of hot water from which it separates in large compact spherical aggregates of needles.  $\text{C}_5\text{H}_6\text{O}_2\text{NSCl}_3$ , calculated S 12.80, N 5.60; found, S 12.56, N 5.56.

The butyric aldehyde compound is recrystallized from 300 cc. of hot alcohol from which it separates on cooling in rectangular plates, m.p.  $167\text{--}168^\circ$ .  $\text{C}_7\text{H}_{13}\text{O}_2\text{NS}$ , calculated, S 18.27, N 8.00; found, S 18.40, N 8.22.

The benzaldehyde compound is recrystallized from 800 cc. of hot alcohol from which it separates in the form of long needles with rectangular ends, m.p.  $159\text{--}160^\circ$ .  $\text{C}_{10}\text{H}_{11}\text{O}_2\text{NS}$ , calculated, S 15.31, N 6.70; found, S 15.27, N 6.81.

The furfural compound is recrystallized from 150 cc. of hot alcohol, m.p.  $125\text{--}126^\circ$ .  $\text{C}_8\text{H}_9\text{O}_3\text{NS}$ , calculated, S 16.07, N 7.04; found, S 16.21, N 7.19.

The cysteine-pyruvic acid compound is extremely easily soluble in water and so is prepared somewhat differently. 13 gm. of cysteine hydrochloride are added to a mixture of 100 cc. of alcohol and 25 cc. of 4 M potassium acetate, and 8 cc. of pyruvic acid are then stirred in. The cysteine rapidly dissolves and a deposit of potassium chloride is left. The mixture is set on ice for a day, is then filtered from the salt, and the filtrate is evaporated to a thick syrup *in vacuo*, the bath temperature being kept below  $60^\circ$ . The syrup is poured out quickly before it crystallizes, the flask is rinsed out with 50 cc. of warm 95 per cent alcohol, and then 50 cc. of absolute alcohol are added, the whole mixture well stirred as the syrup crystallizes, and after digesting on ice for a day the crystalline product is filtered off. The product is recrystallized from 200 cc. of hot glacial acetic acid from which it separates on cooling in tiny crystals, m.p.  $150\text{--}151^\circ$ .  $\text{C}_3\text{H}_4\text{O}_3 \cdot \text{C}_3\text{H}_7\text{O}_2\text{NS}$ , calculated, S 15.31, N 6.70; found, S 15.39, N 6.67.

The interaction of iodoacetic acid with the cysteine in the benzaldehyde compound can be shown as follows: 1 gm. of the benzaldehyde-cysteine compound is dissolved in 12 cc. of water by addition of 1 gm. of sodium bicarbonate, and 1 gm. of iodoacetic

acid is added. After standing a half hour benzaldehyde begins to separate. Let stand overnight to complete the reaction, extract the aldehyde with ether, and add 1 cc. of acetic acid. No precipitate forms, showing that no benzaldehyde-cysteine compound is left. Add HCl to make the solution just acid to Congo red (1 cc. of 6 M HCl is used) and set the clear solution on ice overnight. A crystalline deposit of six-sided plates separates. M.p. 181–182°. These properties together with their analysis show them to be the carboxymethylcysteine previously described (4).  $C_6H_9O_4NS$ , calculated, S 17.86, N 7.82; found S 17.40, N 7.65.

If 0.6 gm. of the benzaldehyde-cysteine compound is dissolved in 60 cc. of 2 M aqueous potassium acetate solution and a solution of 0.6 gm. of phenylhydrazine is added, a crystalline product separates, which after recrystallization has a nitrogen content of 14.24 per cent, while that of benzaldehyde phenylhydrazone is 14.28.

The compound of cysteine with phenylglyoxal previously described (1), as well as that with benzaldehyde, acetylates easily with acetic anhydride with addition of a little anhydrous sodium acetate. After standing 5 hours at room temperature the mixture is evaporated *in vacuo* to a small volume, water is added, and the crystalline product is recrystallized from alcohol.

The acetylated benzaldehyde compound has a melting point of 178–180°.  $C_{12}H_{13}O_3NS$ , calculated, S 12.75, N 5.58; found, S 12.83, N 5.83.

The acetylated phenylglyoxal compound has a melting point of 202°.  $C_{13}H_{13}O_4NS$ , calculated, S 11.46, N 5.02; found, S 10.97, N 5.21.

The cysteine-pyruvic acid addition compound which has both a hydroxyl and an amino group forms a crystalline diacetate by the following treatment. To 5 gm. of the compound suspended in 15 cc. of acetic anhydride 5 cc. of glacial acetic acid and 5 cc. of dry pyridine are added. After a few minutes a clear solution results. After standing for a few hours at room temperature the solution, which has begun to turn a little brown, is evaporated *in vacuo* to a thick syrup. This is rinsed out of the flask with some warm  $CHCl_3$ . A gummy residue separates. After a time the  $CHCl_3$  is decanted and the residue is ground up with absolute alcohol. The gum is converted to a white crystalline solid. This is filtered off and washed with absolute alcohol. The crystals are dissolved in

10 cc. of water, the solution is filtered, and 200 cc. of absolute alcohol are added. After standing on ice for a few hours a crop of tiny rectangular plates separates, m.p. 201–202° with decomposition.  $C_{10}H_{18}O_7NS$ , calculated, S 10.92, N 4.78; found, S 10.56, N 4.33.

The analytical part of this work has been carried out by Mr. G. Bitterlich.

#### SUMMARY

Thioglycolic acid anilide has been shown to combine with simple aldehydes to give 1-hydroxyalkylthio ethers. Although these compounds appear to dissociate readily into their components their acetyl derivatives are relatively more stable. Thioglycolic acid anilide also combines with pyruvic acid, quinone, and isatin.

Cysteine forms easily crystallized condensation products with aldehydes. Some evidence is given that these compounds also dissociate readily, but here again acetylation seems to stabilize the compounds. With pyruvic acid cysteine yields an addition compound which has also been acetylated.

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## A NOTE ON THE DETERMINATION OF IODINE IN BIOLOGICAL MATERIAL

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In a previous communication (1) we described a modification of the Leipter technique (2) for the microdetermination of iodine by means of which we were able to determine amounts of iodine up to 50 micrograms with fair rapidity and with an error not exceeding 10 per cent. With continued use of the method we found it frequently impossible to recover satisfactorily amounts of iodine with which we formerly had obtained good results. At first it was thought that the relatively large excess of phosphorous acid which we employed for reduction of the excess dichromate and of the oxidized iodine might cause incomplete recoveries by reducing some of the iodic acid to hydriodic acid instead of liberating the iodine quantitatively. Attempts were made to control the amount of excess phosphorous acid, but this procedure did not improve the results. It was then found that iodine added as iodide to a reduced digestion mixture containing excess phosphorous acid could be distilled more rapidly than could iodine present as iodate. The difficulty, therefore, lay not in the formation of hydriodic acid, but in the slow reduction of iodic acid by the phosphorous acid. A number of reducing agents were substituted for phosphorous acid but for one reason or another proved undesirable. Antimonious and ferrous salts formed a difficultly soluble precipitate which adhered to the digestion flask. Cuprous oxide and stannous oxide liberated sulfur dioxide from the sulfuric acid and thus prevented the recovery of iodine. Nascent hydrogen was unsatisfactory, and oxalic acid, although giving good iodine recoveries, was deemed undesirable because of the danger attending liberation of carbon monoxide during the distillation. Because of recent pub-

lished work (3) with Leipert's original method, we again tried to use arsenious acid as the reducing agent, this time carefully controlling the temperature during the distillation, but we again found that under the conditions necessary for complete recovery of iodine, a variable but appreciable "blank" was always to be found in the distillate. This finding has been confirmed by other workers.<sup>1</sup>

We then undertook a study of a variety of conditions under which it seemed that it might be possible to increase both the speed of reduction of iodic acid by phosphorous acid and the rate of distillation of the resulting iodine. It was finally found that the addition of more water to the digestion mixture before distillation hastened the reduction of iodic acid, and that the liberated iodine could be distilled rapidly from such a boiling mixture by means of a current of air.

Two other changes in procedure have been made, both of which shorten the time required for a single determination. It has been found that acetic acid and other volatile substances liberated in the digestion may be removed in most cases by a single heating of the digest to 195–200° after oxidation is complete. At this temperature there is no loss of iodine from blood or urine with or without the addition of such compounds as diiodotyrosine and sodium moniodo methyl sulfonate. It also has been found that the addition of sodium bisulfite to the distillate may be omitted without altering the final results. In a series of duplicate determinations on blood, the omission of bisulfite from one of each pair resulted in no significant variation.

The procedure which we recommend is as follows: The material to be analyzed is digested with potassium dichromate and sulfuric acid in the presence of a small amount of cerous sulfate in the manner described in our previous paper (1). There will be no loss of iodine if the digestion is carried out as rapidly as physical conditions will permit. The digestion mixture is then heated over a free flame to a temperature of 195°. If the odor of acetic acid, bromine, or chromyl chloride is still detectable after this treatment, it is necessary partially to cool the digest, to add 10 to 20 cc. of water, and again heat until the temperature reaches 195°. The digest is now cooled and water added, the amount varying with

<sup>1</sup> Personal communications from Dr. Bernard Brodie, New York University, and Dr. Emil J. Baumann, Montefiore Hospital, New York.

the amount of reagents which have been used in the digestion. If 50 cc. or less of concentrated sulfuric acid were used, 50 cc. of water should be added; with more than 50 cc. of sulfuric acid, a volume of water equal to the acid used is required. The digest is now treated with phosphorous acid. We use an 84 per cent solution, by weight, of this reagent, of which about 5 cc. are needed to reduce the average blood digest. Failure to recover any iodine at the end of the procedure usually indicates that an insufficient amount of phosphorous acid was employed; in such a case, an additional 1 or 2 cc. of phosphorous acid and 30 to 40 cc. of water should be added and the mixture redistilled: It has been found that a large excess of phosphorous acid has no effect upon iodine recovery.

After the digestion flask and the receiver are attached to the apparatus, the iodine-containing digest is heated and a slow current of air, just sufficient to mix the digest, is drawn through the apparatus. The air is first washed by bubbling through a 4 inch column of dilute sodium hydroxide. When the digest begins to boil, the suction is increased so that the air current produces a rapid, but not violent, bubbling through the receiver (50 to 75 liters per hour). The boiling and aeration are continued for 15 minutes if less than 20 micrograms of iodine are present, or for 30 minutes with amounts of iodine from 20 micrograms to 1 mg. With quantities of iodine exceeding 50 micrograms; a second alkaline receiver attached in series with the first is required. At the end of the distillation, from 30 to 40 cc. of liquid should have condensed in the first receiver and the temperature of the boiling digest should not exceed 150°. The temperature of the digest is ascertained by means of a short thermometer of 110–170° range attached to the central aeration tube by means of a small rubber band. If the temperature has reached 150° before the distillation is finished, the flame under the flask should be lowered so that a temperature of 145–150° is maintained during the remainder of the aeration period. The distillate is made acid without previous treatment with sulfite, brominated, evaporated, and treated as described in our previous paper. For amounts of iodine of 10 micrograms or less, the solution should be evaporated to a final volume of 2 cc.; with amounts of iodine as large as 1 mg., the volume may be as much as 25 cc.



There are some further points in connection with the procedure which merit brief discussion. It has been found by Harvey (4), and confirmed by the present writers, that the amount of iodate reacting with KI and starch in a volume of 1 cc. must be equivalent to 0.06 microgram of iodine in order that the color be visible. This fact must be taken into consideration in the calculation of results, both in standardizing thiosulfate solutions and in the determination of the iodine content of unknown solutions. To obtain an absolute factor for the standard thiosulfate solution, the equation used is:

$$1 \text{ cc. Na}_2\text{S}_2\text{O}_3 = \frac{\text{micrograms I}_2 \text{ as KIO}_3 - 0.06 V}{\text{cc. thiosulfate used}}$$

where  $V$  equals the volume of the solution in cc., after titration. In the determination of unknowns, the following calculation is made:

$$\text{Micrograms I}_2 = (\text{factor} \times \text{cc. thiosulfate}) + 0.06 V$$

The volume correction may be omitted entirely if the thiosulfate is standardized against an amount of iodate containing within 10 per cent of the amount of iodine to be determined in the unknown material. In such a case the standardization and subsequent titrations must be carried out in the same volume of liquid. It is expedient to standardize thiosulfate solutions in the presence of approximately that amount of sodium or potassium sulfate which will be present in the final titration of unknowns, since it has been found that the presence of salts influences to some extent the sensitivity of the titration.

When KI is added in the final titration, care must be taken to keep the amount added within a fairly narrow range, as it appears that significant variations in the titration figure may be produced by using uncontrolled amounts of this reagent. The following figures show the effect of varying amounts of KI on the titration of 1 cc. of 0.0001 N KIO<sub>3</sub>.

KI added mg.	Thiosulfate used cc.
5	0.088
10	0.094
20	0.099
50	0.105

The KI may be added either in solution or in the dry form; we prefer the latter because the instability of KI solutions necessitates their daily preparation, with resulting inconvenience and needless waste. The finely granular KI may be measured by means of a small aluminum spoon, constructed to hold approximately 20 mg., with sufficient accuracy to insure consistent titration values.

Employing the method as described, we have determined amounts of iodine ranging from 0.2 to 1000 micrograms in over 100 samples of material with an error not exceeding, and usually less than, 10 per cent.

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# COPPER CATALYSIS OF THE OXIDATION OF THIOL ACIDS AS A BASIS FOR THE MICRODETERMINATION OF COPPER

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In an attempt to find a method which might be more accurate than Warburg's cysteine oxidation (1) for the determination of small amounts of copper, it was found that thiol acids such as thioglycolic, thiolactic, and thiomaleic acids (contrary to cysteine (2)) were oxidized by air in 0.1 to 1 N hydrochloric acid solution in the presence of traces of copper. The oxidation of both thioglycolic and thiolactic acids to the disulfide acids could be followed by a simple iodine titration, but thiomaleic acid, like cysteine (3), was oxidized beyond the disulfide stage.

The rate of oxidation was about the same for the three thiol acids. The amount oxidized was proportional to the time for the first 40 to 50 per cent, after which the rate slowed down. Just as with the cysteine oxidation (4), there was a certain critical (very small) copper concentration, which gives the maximal rate of oxidation. At concentrations below this critical value the rate of oxidation was, within certain limits, proportional to the copper concentrations. Increase of the copper concentration beyond the critical value has very little influence. In a similar way the rate of oxidation increases with the thiol acid concentration until this concentration reaches an optimal value, after which increasing thiol acid concentration has a depressing influence. In thioglycolic acid solutions 0.5 N in HCl the critical copper concentration is around  $3 \times 10^{-5}$  M (= 2 mg. of Cu per liter) and the optimal thiol acid concentration about 0.05 M. Smaller hydrochloric acid concentrations give greater rates of oxidation, but at the same time the critical copper concentration falls very much.

Thioglycolic acid solutions with a copper content around the critical were always quite clear, but increase of the copper content to only a few times this value caused turbidity, owing to the very sparingly soluble cuprous thiol compound.

### *Specificity of the Catalysis*

The influence of other metals and some anions was tried in 0.025 M thioglycolic acid solutions, 0.25 N in HCl. Manganous, ferrous, cobaltous, nickel, zinc, cadmium, lead, chromic, and bismuth ions in concentrations from 0.001 to 0.004 M showed no

TABLE I

### *Determination of Copper in Human Serum*

Thiol acid concentration, 0.0245 M; HCl, 0.25 N; total volume of solutions, 4 cc.

Added substance	Iodine consumption after 18 hrs	Thiol acid oxidation
	cc. 0.01 N solution	per cent
None.....	9 71	0
0.000125 mg. Cu (as CuSO <sub>4</sub> ).....	8.74	10.0
0.00025 " " .....	7.23	25.6
0.0005 " " .....	4.50	53.7
0.001 " " .....	1.47	84.9
0.002 " " .....	0.35	96.5
0.25 cc serum.....	6.81	29.8
0.50 " " .....	4.86	50.0
0.25 " " + 0.00025 mg. Cu .....	4.08	58.0

catalysis of their own and had practically no influence on the catalysis caused by copper in a concentration of  $10^{-6}$  M. Mercuric ions gave a crystalline precipitation in approximately 0.0003 M solution and ferric ions oxidized 1 equivalent of thiol acid but both had no other influence on the oxidation. Of the anions tested nitrate, sulfate, phosphate, and acetate, in concentrations around 0.01 M, had no influence. Fluoride in 0.01 M solution increased the catalysis about 25 per cent, while sulfocyanide in the same concentration had a strong inhibiting influence, cutting down the rate of oxidation about 50 per cent.

Unfortunately it was not possible to reproduce the individual experiments with an accuracy higher than 10 to 15 per cent. It was, therefore, not possible to work out a method which was better than Warburg's cysteine oxidation. The experiments, however, have some interest, for they indicate a simple iodometric method for the estimation of small amounts of copper of the order of  $10^{-4}$  mg. The method requires neither special apparatus nor the use of the more expensive cysteine.

In Table I is given an example of such copper determination in human serum with thioglycolic acid. For the experiments a vacuum-distilled thioglycolic acid, which could be kept several months without being oxidized, as a 0.1 M solution in 1 N HCl was used. The experiments were made with small conical flasks which were filled to only a small extent. The flasks with contents were shaken for the proper length of time in a shaking machine at 25°, after which the unoxidized thiol acid was determined by iodine titration.

From Table I it can be estimated that the serum examined had a copper content of a little more than 1 mg. per liter. This is in agreement with what Warburg and Krebs (5) and Bjerrum and Henriques (6) find by the method of cysteine oxidation. In cow's milk in a similar way a copper content from 0.05 to 0.07 mg. per liter was found, in agreement with the latest determinations in the literature (7).

The author wishes to express his thanks to Professor Vald. Henriques for his kind interest in the work.

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## FURTHER STUDY OF THE GROWTH EFFECT OF THE RESIDUE REMAINING AFTER ALCOHOLIC EXTRACTION OF YEAST\*

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An increasing amount of evidence that the so called vitamin B complex contains other components than vitamins B and G has accumulated. Many of these additional factors have been reported, but there has been little done in the way of correlation or confirmation of the different findings. The lack of standardization of methods and materials may explain in part the diverse interpretations of the results of vitamin B complex studies and the disparity between the "third factors" reported. Certainly, the existence of so many distinct factors as have been claimed is open to question.

Among the claims for a so called third factor in the vitamin B complex is that of Williams and Lewis (1930) (13) from this laboratory. They reported that when bakers' yeast was extracted four times with progressively increasing strengths of boiling alcohol, a growth factor for rats remained in the insoluble residue, Fraction R. This factor appeared to be qualitatively different from vitamins B and G, since the yeast extract or fractions thereof which contained these vitamins, even in 4 "gm. yeast equivalents" (*cf.* (13) p. 280) each, failed to supplement the vitamin B complex-free diet so that anything like normal growth was obtained; whereas, the extract or its fractions plus yeast residue in amounts of 1 gm. yeast equivalent each promoted growth equal to that obtained

\* The experimental data in this paper are taken from the dissertation submitted by Marion Reinhardt Rymer to the Graduate School of the University of Colorado in partial fulfilment of the requirements for the degree of Doctor of Philosophy.



with 1 gm. of yeast. Moreover, 2 gm. yeast equivalents of the residue failed to induce growth, and many of the animals of this group developed lesions of the eyelids and nose. The factor was shown to be thermolabile and insoluble in hot or cold water, alcohol, and acids. Later reports from this laboratory (8, 9) showed it to be alkaline heat-labile, but stable in acid solution.

Following the report of Lewis and Rymer (9) at the Cincinnati meeting of the American Society of Biological Chemists, Griffith (7) suggested that a quantitative lack of vitamin G in the diet might explain the apparent existence of many of the so called third factors. The present paper deals with our attempts to determine whether the results reported by Williams and Lewis (13), which have been repeatedly confirmed by us, could have been due to a deficiency of vitamin G in the yeast extract.

#### EXPERIMENTAL

*Procedure*—In general, the experimental procedure was similar to that reported earlier (13). Albino rats, 28 to 32 days old, were placed in individual cages with false floors and given the Sherman-Spohn basal diet, modified by the replacement of 2 per cent of the corn-starch with powdered agar-agar. Following a depletion period of 10 to 14 days on the basal diet<sup>1</sup> alone, the various supplements to be tested were given daily.

Five preparations were used as vitamin supplements. Unfractionated yeast<sup>2</sup> extract and yeast residue were made according to the method outlined by Williams and Lewis (13). A rice polishings<sup>3</sup> concentrate, which was relatively rich in vitamin B and poor in vitamin G as a result of precipitation in 90 per cent alcohol, was prepared according to the method of Evans and Lepkovsky (5). Two preparations rich in vitamin G and poor in vitamin B were

<sup>1</sup> Rats placed on this diet at 28 to 32 days of age gained little or no weight; later, they lost weight and died within 23 to 30 days in a state of great weakness.

<sup>2</sup> Fleischmann's yeast was kindly supplied by Standard Brands, Inc., New York. According to Dr. C. A. Smith of this company, the yeast was non-irradiated and contained from 12 to 15 Chase and Sherman units of vitamin B and the same number of Bourquin and Sherman units of vitamin G (personal communication).

<sup>3</sup> The rice polishings were kindly furnished by the Louisiana State Rice Milling Company, Abbeville, Louisiana.

used: dried autoclaved liver prepared according to the method of Graham and Griffith (6); and egg white concentrate, according to Chick, Copping, and Roscoe (3). The total amount of any one preparation needed for a series of animals was calculated and made up at the beginning of the experiment.

*Animal Experiments*—As a preliminary experiment to test the validity of Griffith's criticism that a deficiency of vitamin G in the yeast extract might explain our earlier results, the dosage of yeast extract was increased 10-fold for three of seven litter mate female rats which had received 1 gm. yeast equivalent of yeast extract per day for 40 days. Their growth rate increased from 0.68 gm. per day to 1.93 gm. per day for the 30 days subsequent to increasing the amount of extract. This was about the normal rate of gain for females which received 1 gm. yeast equivalent of yeast extract plus 1 gm. of yeast residue (*cf.* Table I, Group 21). After the remaining four rats of this group had served for 20 days as controls for the above experiment, 1 gm. of dried autoclaved liver was added to each rat's daily supplement. From an average daily growth of only 0.70 gm. for the preceding 60 days, the growth rate increased to 1.42 gm. for the following 30 days. Thus, it was evident that some of the growth factor or factors necessary to supplement 1 gm. yeast equivalent of the yeast extract were also present in the yeast extract and in dried autoclaved liver.

Next, an attempt was made to determine whether the yeast residue would still stimulate further growth when 1 gm. yeast equivalent of yeast extract was supplemented by substances rich in vitamin G. The supplements fed and the results of these experiments are presented in detail in Table I. In general, when rich sources of vitamin G, such as egg white concentrate (Group 22) or dried autoclaved liver (Group 24), were added to the diet in addition to 1 gm. yeast equivalent of yeast extract, the growth of the animals was markedly increased over that obtained with 1 gm. yeast equivalent of the yeast extract alone (Group 20). When yeast residue was used in addition to these supplements (Groups 23 and 25), these growth rates were still further increased so that they were slightly superior to that of the controls (Group 21). When 5 or 10 gm. yeast equivalents of yeast extract (extra vitamins G and B) were fed (Groups 26 and 28), a normal growth rate was obtained. Even this was increased slightly when yeast residue

was added (Groups 27 and 29). The evidence from this series of animals shows that a large part, but not all, of the increased growth which had been attributed to a "third factor" in the yeast residue was in reality due to its content of vitamin G.

TABLE I

*Growth of Rats on Vitamin B Complex-Free Diet Supplemented As Indicated*

Group No.	Supplements per rat per day	Daily gain of individual rats (50 days)		Average daily gain	
		Males	Females	Males	Females
		gm	gm	gm.	gm.
20	1 gm. yeast equivalent yeast extract	1.80, 1.70, 1.62, 1.14	0.72, 0.62, 0.66, 0.96, 1.48	1 57	0 89
21	Same + 1 gm. yeast residue	2.60, 3.04, 3.14, 2.80, 2.50, 3.02, 3 12	1.94, 2.24, 1.94, 1.94, 2.04	2 89	2 02
22	1 gm. yeast equivalent yeast extract + 5 gm. equivalents egg white	2.40, 2.14, 2 36		2 30	
23	Same + 1 gm. yeast residue	3.28, 3.20, 3.46		3 31	
24	1 gm. yeast equivalent yeast extract + 1 gm. autoclaved liver	2.56, 2.68, 2.25	1.64, 2.20	2 50	1 92
25	Same + 1 gm. yeast residue	2.64, 3.16, 3.26, 3.84	2.06	3 23	2 06
26	5 gm. yeast equivalents yeast extract	3.24, 3.12, 2.21, 3.14	2 30	2 93	2 30
27	Same + 1 gm. yeast residue	3.06, 3.20, 3.28, 3.10	2.58	3 16	2 58
28	10 gm. yeast equivalents yeast extract	2.72, 3.05, 3.06	2.26, 2.14	2 94	2 20
29	Same + 1 gm. yeast residue	3.28, 3.10, 3.16	2.20, 2.12	3 18	2 16

In an attempt to determine whether the remaining activity of the yeast residue was due to the presence of vitamin B, various amounts of this vitamin were supplied as rice polishings concentrate with and without the yeast residue. Vitamin G was furnished in dried autoclaved liver. The details of this experiment

are presented in Table II. In general, the growth rates of all of the animals in this series are very similar and show no constant variations which can be correlated with the quantitative or qualitative differences in the supplements fed. The slightly lower growth rates of the animals in this series as compared with that of the controls (Group 21, Table I) may have been due to the extremely hot weather which prevailed throughout this experiment. The data indicate that when vitamin B and vitamin G are fed in adequate amounts as dried autoclaved liver and rice polishings concentrate, respectively, the yeast residue has no effect in stimulating further growth.

TABLE II

*Growth of Male Rats on Vitamin B Complex-Free Diet Supplemented As Indicated*

Group No	Supplements per rat per day	Daily gain of individual rats (50 days)	Average daily gain gm.
30	1 gm. equivalent rice polishings + 1 gm. autoclaved liver	2.58, 2.80, 2.34, 2.28, 2.34, 2.82, 2.48	2.52
31	Same + 1 gm. yeast residue	3.08, 2.16, 2.84, 2.38, 2.92, 3.50, 3.10, 2.32	2.79
	2.5 gm. equivalents rice polishings + 1 gm. autoclaved liver	1.96, 2.20, 2.84, 2.82, 2.52, 3.34, 2.76, 2.30	2.59
33	Same + 1 gm. yeast residue	2.54, 2.48, 2.58, 2.30, 2.08, 2.72, 2.10, 3.28	2.51
34	5 gm. equivalents rice polishings + 1 gm. autoclaved liver	2.26, 3.22, 2.24, 2.92, 2.56, 2.86	2.68
35	Same + 1 gm. yeast residue	2.74, 2.46, 3.08, 2.32, 2.96, 2.36, 2.20	2.59

## DISCUSSION

The experiments presented in this report indicate that the growth-promoting effect of the yeast residue, which Williams and Lewis (13) had ascribed to a "third factor" of the vitamin B complex, is in reality due for the most part to the presence of vitamin G, since the dried autoclaved liver and egg white concentrate, both rich sources of vitamin G, can largely replace the yeast residue. Furthermore, the data show that normal growth occurs when either rice polishings concentrate or yeast residue plus yeast extract (1

gm. yeast equivalent each) is given with autoclaved liver as a supplement to the basal diet, but that this small amount of yeast extract alone does not completely augment autoclaved liver. Thus, it may be concluded that the effectiveness of the yeast residue in these experiments is due to some factor which is also present in the rice polishings concentrate. Since the rice polishings concentrate is known to be a rich source of vitamin B, it seems logical to ascribe a part of the growth-promoting effect of the yeast residue to the presence of vitamin B. Accordingly, the value of the yeast residue, when added to yeast extract, may be explained on the ground that it supplies vitamins B and G rather than because it contains a "third factor."

The cause of the low potency of the yeast extract in vitamins B and G needs some consideration. Since the original yeast contained 12 to 15 Sherman units of both of these vitamins, they must have been either only partially extracted or partially destroyed in the extraction process. In the light of recent reports in the literature, it appears that both possibilities were operative. Chick and Roscoe (1929) (4), Chick and Copping (1930) (2), Narayanan and Drummond (1930) (10), Evans and Lepkovsky (1930-31) (5), Sherman and Sandels (1930-31) (11), Stiebeling and Alleman (1933) (12), and Booher (1933) (1) found in different types of experiments that vitamin G is either insoluble, or only partially soluble, in alcohol of relatively high concentrations, or that vitamin G is only partly extracted from its various substrates. In these same papers, Chick and Roscoe, Chick and Copping, Sherman and Sandels, and Stiebeling and Alleman presented evidence to show that vitamin G is destroyed by alcohol or some other factor during the extraction process. With reference to vitamin B, it is possible that the repeated and prolonged heating during the extraction of the yeast and the concentration of our yeast extract may have caused partial destruction of vitamin B. Moreover, it should be noted that Stiebeling and Alleman (12) found vitamin B to be incompletely extracted from skim milk powder by 80 per cent alcohol.

Even though the results of our experiments show that the yeast residue exerts no beneficial effect upon the growth of rats when adequate amounts of vitamins B and G are supplied, we do not feel justified in drawing the unqualified conclusion that the yeast

residue contains no other growth factors than those of vitamin B and vitamin G. We are working with very complex, impure substances, and it must be borne in mind that the liver, rice polishings concentrate, or yeast extract may have contained the same "third factor" that was postulated for the yeast residue. The final solution of the problem of whether a "third factor" actually exists lies in the use of preparations of vitamins B and G which are free from traces of other factors.

" SUMMARY

In a further study of the alleged third factor reported by Williams and Lewis to be present in the yeast residue which remains after alcoholic extraction of yeast, experimental evidence has been obtained which indicates that the growth-promoting ability of the yeast residue is due in large measure to the presence of vitamin G and vitamin B. Although our experiments do not disprove the existence of this third factor, the postulation of its presence in yeast to explain the effect of the yeast residue on the growth of rats seems hardly necessary in the light of our experimental data.

We are grateful to Dr. Charles A. Rymer and Mr. Leland C. Messex for able assistance during the progress of our experimental work.

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# THE PROTEIN CONTENT OF HUMAN PAROTID SALIVA

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The analyses reported here concern the protein content of the human parotid secretion and the changes in concentration at different rates of secretion. That the rate of secretion affects the composition of the saliva has not been emphasized often, although the fact has long been known (1). In only a few reports of analyses of various salivary constituents is the rate considered an important factor. Determinations of salivary proteins with the currently accepted methods are rare. Organic solids in mixed and parotid saliva are reported by Hubbell (2) and others (3, 4), but no definite correlation between rate of secretion and the solid content was noted.

## *Method*

The parotid saliva was collected uncontaminated by other secretions by means of a small cup held securely over the mouth of the parotid duct by a suction ring, the secretion being allowed to flow through a tube from the cup under its own pressure. For different rates of secretion several stimuli were used: paraffin, lemon, ether, 1 per cent tartaric acid, 1 per cent citric acid. The parotid secretion thus obtained is a clear watery fluid quite unlike the ordinary mixed saliva. When, during the collection of a set of samples, the stimulus was changed, at least 2 cc. of saliva were collected with the new stimulus and discarded to allow the collecting apparatus to be cleared of saliva produced for the preceding sample. This method was found to be sufficient to obtain consistent checks when changing to different rates.

Samples were analyzed for total and non-protein nitrogen according to Howe's Kjeldahl method (5), slightly modified as to the size of the samples used. All analyses were made in duplicate



and the digested mixtures were distilled with steam into 0.02 N hydrochloric acid. Various qualitative tests were made as indi-

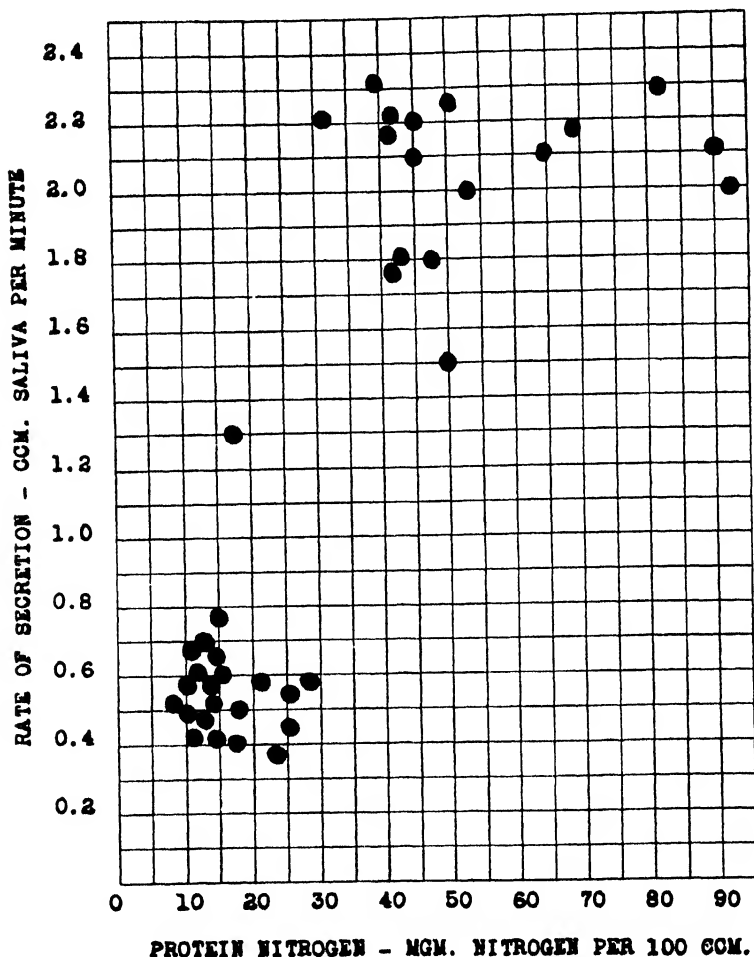


FIG. 1. Protein nitrogen of parotid saliva

cated later. The saliva for these experiments was collected from a male aged 30 years and in good health.

Since it is possible that successive specimens might change in

composition merely from long continued stimulation independently of the rate of secretion, the specimens were taken by applying the stimuli in four different sequences: rapid, slow, rapid; slow, rapid, slow; rapid, rapid, rapid; slow, slow, slow. This variation caused no significant difference in samples of the same rate of secretion but collected in a different order.

#### DISCUSSION

The results of the parotid saliva analyses are plotted in Fig. 1, and show a fairly consistent increase of protein nitrogen concentration with increase of rate.

As determined by qualitative tests, the protein is principally albumin, but the quantity is so small that accurate analyses after fractional precipitation did not seem possible. The usual tests for mucoprotein (6) were negative. In contrast, mixed saliva contains considerable mucin, and its protein content as indicated by a few analyses changes but little with change of rate of secretion. For example, mixed saliva produced at a rate of 2 cc. per minute contains an average of 40 mg. of protein nitrogen per 100 cc. and that at 9 cc. per minute an average of 30 mg., as compared with the marked changes in parotid saliva with much less change in rate, as shown in Fig. 1.

#### SUMMARY

The protein nitrogen concentration of human parotid saliva increases progressively with increased secretion. With the stimuli used in these experiments the range is from 11 mg. of protein nitrogen per 100 cc. of saliva at a rate of 0.5 cc. per minute to an average of 58 mg. of nitrogen at rates of about 2 cc. per minute.

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## CALCULATION OF ISOELECTRIC ZONES AND ISOELECTRIC POINTS

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### *Isoelectric Zones of Simple Ampholytes*

The dissociation of simple ampholytes is clearly depicted by the well known curves of Michaelis (7), who plotted against pH the fraction,  $\rho$ , of the total ampholyte which is in the undissociated or zwitter ion state. These curves are bell-shaped, having maxima at the isoelectric point, and each curve is symmetrical about a vertical axis through the isoelectric point. The curves show that the maximal value of  $\rho$  is close to unity when the product,  $k_a k_b$ , of the classical acid and basic dissociation constants is small, and that in such cases  $\rho$  is practically constant over a broad isoelectric zone of pH values. If  $k_a k_b$  is sufficiently large, the isoelectric point is more sharply defined and the maximal value of  $\rho$  is noticeably less than 1; that is, a considerable fraction of the ampholyte exists in the form of cations and anions, even at the isoelectric point. It is possible to extend Michaelis' treatment by calculating the breadth of the isoelectric zone.

The advantages of writing all ionization constants of an ampholyte as acid constants have been set forth by Adams (1) and Brönsted (2). For a substance which may exist as  $R^+$ ,  $R$ , or  $R^-$ , such ionization constants may be defined as

$$K_1 = (H^+ \cdot R)/R^+, \quad K_2 = (H^+ \cdot R^-)/R \quad (1)$$

If  $I$  is the value of  $H^+$  at the isoelectric point, which is determined by the condition that  $R^+$  and  $R^-$  are equal, or  $\rho$  is at a maximum, the isoelectric point is given by the equation

$$I^2 = K_1 K_2 \quad (2)$$

The value of  $\rho$  at any point is given by

$$\frac{1}{\rho} = 1 + \frac{H^+}{K_1} + \frac{K_2}{H^+} \quad (3)$$

while its maximal value, at the isoelectric point, is given by

$$1/\rho_{\max.} = 1 + 2\sqrt{K_2/K_1} \quad (4)$$

Equations 2, 3, and 4 were derived by Michaelis, who used the classical acid and basic constants.

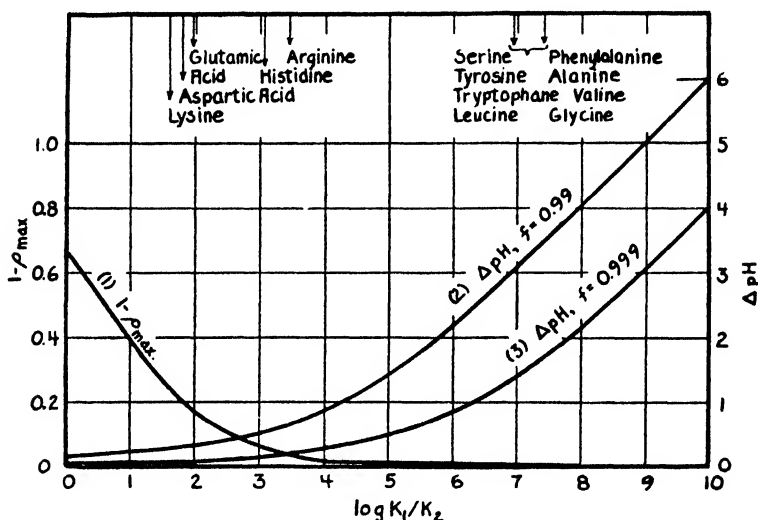


FIG. 1. Curve 1 shows values of  $1 - \rho_{\max}$ , the fraction of a simple ampholyte ionized as cations and anions at the isoelectric point, as a function of the logarithm of the ratio of its dissociation constants. Curves 2 and 3 show  $\Delta pH$ , the breadth of the isoelectric zone of a simple ampholyte, so defined that the electrically neutral fraction shall have 99.0 and 99.9 per cent of its maximum value.

Since the second ionization constant of an acid is never greater than the first, Equation 4 implies that  $\rho_{\max.}$  is never less than one-third, or that no more than two-thirds of any ampholyte can exist in the form of anions and cations at the isoelectric point. The dependence of this ionized fraction,  $1 - \rho_{\max.}$ , on the ratio of the two acid constants is shown by Curve 1 of Fig. 1.

In order to calculate the breadth of the isoelectric zone, it is necessary to define the latter in such a way as to meet practical requirements. It is not sufficient to postulate that  $\rho$  shall have its maximal value, because this leads to a definite isoelectric point even in those cases where there is, for all practical purposes, a wide isoelectric zone. This zone may be defined as the difference in pH between two points on opposite branches of the  $\rho$ -pH curve where  $\rho$  has the same value, which is specified as a definite large fraction,  $f$ , of its maximal value. This definition of  $f$ , with Equations 3 and 4, gives

$$f = \frac{1 + 2\sqrt{K_2/K_1}}{1 + \frac{H^+}{K_1} + \frac{K_2}{H^+}} \quad (5)$$

Equation 5 may be simplified by introducing a new variable,  $q$ , which is defined by

$$q = \frac{1-f}{f} \sqrt{\frac{K_1}{K_2}} + \frac{2}{f} \quad (6)$$

Equations 2, 5, and 6 then yield the relation

$$q = \frac{H^+}{I} + \frac{I}{H^+} \quad (7)$$

which may be solved for  $H^+$

$$H^+ = \frac{qI}{2} (1 \pm \sqrt{1 - (4/q^2)}) \quad (8)$$

The breadth of the isoelectric zone in pH units is given by the difference between the logarithms of the two values of  $H^+$  obtained from Equation 8, or

$$\Delta pH = \log \frac{1 + \sqrt{1 - (4/q^2)}}{1 - \sqrt{1 - (4/q^2)}} \quad (9)$$

The breadth of the zone thus depends only on the values of  $f$  and of the ratio of the dissociation constants. By assuming a value for  $f$  which will satisfy experimental requirements, one may obtain  $q$  by Equation 6 for any given ratio of the constants and then get the breadth of the isoelectric zone by Equation 9.

The results of such calculations are shown in Fig. 1, Curves 2 and 3, in which the breadth of the isoelectric zone is plotted against the logarithm of  $K_1/K_2$ ,  $f$  being taken as 0.99 or 0.999. The figure also shows the logarithms of the ratios of the constants determining the isoelectric point for several typical amino acids, as tabulated by Cohn (3). Even for the dibasic or diacidic amino acids the breadth of the isoelectric zone may be several tenths of a pH unit, while for the simple amino acids the breadth may be several pH units. Apparently the precise location of the isoelectric point of the latter substances can have little experimental significance.

#### *Isoelectric Points of Multivalent Ampholytes*

It is known that proteins, as well as the multivalent peptides recently studied by Greenstein and Joseph (4), may have sharp isoelectric points. This must be a consequence of the fact that their ionization constants are relatively close together. In such a case, however, Equation 2 may no longer suffice for the calculation of the isoelectric point. An exact equation for a multivalent ampholyte was given by Levene and Simms (6), who used titration constants which differ from the true dissociation constants when the latter are not far apart. As Simms (8) later pointed out, less complex equations are obtained if the true dissociation constants are used.

The derivation of a general formula may be illustrated by considering the case of a dibasic, diacidic ampholyte which may exist as  $R^{++}$ ,  $R^+$ ,  $R$ ,  $R^-$ , and  $R^{--}$ . Its dissociation constants are

$$K_1 = \frac{H^+ \cdot R^+}{R^{++}}, \quad K_2 = \frac{H^+ \cdot R}{R^+}, \quad K_3 = \frac{H^+ \cdot R^-}{R}, \quad K_4 = \frac{H^+ \cdot R^{--}}{R^-} \quad (10)$$

while its isoelectric point is defined by the relation

$$2R^{++} + R^+ = R^- + 2R^{--}$$

or

$$\frac{2I^2}{K_1 K_2} + \frac{I}{K_2} = \frac{K_3}{I} + \frac{2K_3 K_4}{I^2}$$

This may be written

$$I^2 = K_2 K_3 \frac{1 + (2K_4/I)}{1 + (2I/K_1)} \quad (11)$$

Evidently Equation 11 will reduce to the form of Equation 2 if  $I$  is much less than  $K_1$  and much greater than  $K_4$ , which will often be the case. If the constants are not widely separated, Equation 11 may be used by substituting  $\sqrt{K_2 K_3}$  for  $I$  in the right member. If the value of  $I$  obtained differs appreciably from that assumed, the approximation may be repeated until the desired accuracy has been reached. A formula which may be reduced to Equation 11 was given by Simms (8), but the form given here seems to show more clearly the effect of the additional constants.

For an ampholyte capable of producing ions of still higher valence, Equation 11 may be generalized to read

$$I^2 = K_n K_{n+1} \frac{1 + \frac{2K_{n+2}}{I} + \frac{3K_{n+2}K_{n+3}}{I^2} + \dots}{1 + \frac{2I}{K_{n-1}} + \frac{3I^2}{K_{n-1}K_{n-2}} + \dots} \quad (12)$$

Here the constants are again numbered in the order of decreasing magnitude, with  $n$  referring to the highest valence of any cation of the ampholyte. It seems possible that Equation 12 may be of use in interpreting the ionization of proteins, which have many dissociation constants not widely separated.

The equations of this paper have been given, for simplicity, in the classical form, with concentrations rather than activities. Equations in this form are exact if each  $K$  is an apparent constant which includes all of the activity coefficients and may therefore vary with the ionic strength of the solution. If the true constants are used, Equation 2 must be written

$$I^2 = K_1 K_2 \frac{\gamma_{R^+}}{\gamma_{H^+}^2 \gamma_{R^-}} \quad (2, a)$$

In Equations 11 and 12 the only activity coefficients which may need to be considered are those indicated in Equation 2, a; the effect of the additional constants is so slight that it will make practically no difference whether true or apparent values are used.

### *Methods of Expressing Isoelectric Points*

In the preceding discussion the isoelectric point,  $I$ , has been taken as the concentration of hydrogen ions in any solution in



which there are equivalent amounts of those positive and negative ions of the ampholyte which are formed by the addition or loss of the hydrogen ion. As Sørensen and coworkers (10) pointed out, this isoionic condition is identical with the isoelectric condition only if the ampholyte does not combine with ions other than hydrogen and hydroxyl. Sørensen *et al.*, however, defined the isoelectric and isoionic points in terms of the activity of the hydrogen ion. If this is done, Equation 2, a becomes

$$(I\gamma_{H^+})^2 = K_1 K_2 \frac{\gamma_{R^+}}{\gamma_{R^-}} \quad (2, b)$$

or

$$pI' = \frac{1}{2} (pK'_1 + pK'_2) \quad (2, c)$$

if  $pI'$  is the value of  $paH$  at the isoelectric point and  $pK'_1$  and  $pK'_2$  are the negative logarithms of the usual apparent dissociation constants, as determined from  $paH$  measurements. The isoelectric points of amino acids have generally been calculated by Equation 2, c.

This method of expressing isoelectric points in terms of  $paH$  or  $pH$  is open to objection because these quantities are not thermodynamically defined and their values may be obtained only by arbitrary assumptions. Different assumptions lead to different scales of  $pH$  values, and Sørensen himself has advocated scales of  $pH$  and  $paH$  differing by 0.04 unit. Moreover, repeated measurements with a single solution may yield different values because a liquid junction is not readily reproducible.

If the isoelectric point is to have a quantitative theoretical significance, it should be described in more definite terms. This could be done by means of hydrogen ion concentration, since the ratio of activity coefficients in Equation 2, a is thermodynamically defined, although the value of this ratio is unknown for most amino acids.

A new and thermodynamically sound method of describing isoelectric points may be based on the electromotive force of a cell without liquid junction. If a solution containing chloride ion in known concentration is placed in such a cell, provided with a hydrogen electrode and a silver-silver chloride electrode, the measure-

ment gives a value for  $m_{H^+ \gamma_{H^+} \gamma_{Cl^-}}$ , a quantity which has been proposed as a unit of acidity (5). An isoelectric point in this unit is related to the dissociation constants of the ampholyte by the equation

$$(I \gamma_{H^+} \gamma_{Cl^-})^2 = K_1 K_2 \frac{\gamma_{R^+} \gamma_{Cl^-}^2}{\gamma_{R^-}} \quad (2, d)$$

By extrapolating  $I \gamma_{H^+} \gamma_{Cl^-}$  to zero ionic strength, it should be possible to obtain for each ampholyte a value of  $I$  which will be a true isoelectric constant, equal to  $\sqrt{K_1 K_2}$ . Such an extrapolation is possible even when the dissociation constants are unknown, as Smith (9) has shown for the case of egg albumin. Her extrapolated value, however, may be burdened with the errors inherent in the pH scale, and such errors would be avoided by the use of the method just described.

The writer wishes to acknowledge his indebtedness to Dr. Leslie F. Nims for helpful discussion of the equations of this paper.

#### SUMMARY

A method is given for calculating the breadth of the isoelectric zone of a simple ampholyte as a function of the ratio of its dissociation constants. A general formula is given for the isoelectric point of a multivalent ampholyte in terms of all of its dissociation constants. The possible application of these equations to the dissociation of amino acids and proteins is suggested. The expression of isoelectric points in thermodynamically defined units is advocated.

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## DEUTERIUM AS AN INDICATOR IN THE STUDY OF INTERMEDIARY METABOLISM

### VI. SYNTHESIS AND DESTRUCTION OF FATTY ACIDS IN THE ORGANISM\*

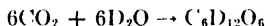
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(Received for publication, March 23, 1936)

In previous publications we have shown that deuterium can be used to label foodstuffs so that their transportation and conversion into other substances could be followed (1-5) (see also (6)). We present in this paper a general method for determining the rate of synthesis and destruction of individual constituents of an organism. Organic substances synthesized in an aqueous medium containing heavy water will, in general, contain deuterium. The rate of appearance of deuterium in the organic compound will then be proportional to the rate of synthesis. In this communication we describe the application of this method to the fatty acids.<sup>1</sup>

Plants synthesize organic substances from CO<sub>2</sub> and water. When they are kept in heavy water instead of ordinary water, the equation for the synthesis of sugar will be



The result would be a hexose in which all of the hydrogen atoms are present as the heavy isotope, deuterium. The same would hold for the fatty acids synthesized by plants; stearic acid would have the formula C<sub>18</sub>D<sub>36</sub>O<sub>2</sub>.<sup>2</sup> All deuterium atoms in such fatty

\* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

<sup>1</sup> The rate of synthesis of cholesterol in animals has also been determined in this manner. The results will be described in a future publication.

<sup>2</sup> Plants do not grow well in concentrated D<sub>2</sub>O (7, 8). In dilute D<sub>2</sub>O a mixture of fatty acids with the corresponding isotopic ratio would result.

acids, with the exception of the one attached to the carboxyl group ( $\text{—COOD}$ ), would be attached directly to carbon and therefore would be stable. By dissolving or heating with acids or alkalis in ordinary water, they would not be replaced by the protium atoms of the solvent.<sup>3</sup> Reitz and Bonhoeffer (8) have grown green algæ in heavy water of various concentrations. They observed that the dry substance of these plants contained both exchangeable and non-exchangeable deuterium.

The animal organism is not able to carry out so fundamental a synthesis. Its synthetic processes consist largely in the coupling of small organic molecules. When these reactions proceed in a medium of heavy water instead of ordinary water, deuterium will, in general, enter the reaction and may appear in stable form (bound to carbon) in the end-products. The number of deuterium atoms in such substances will in most cases be smaller than in substances synthesized under the same conditions by plants from  $\text{CO}_2$  and  $\text{D}_2\text{O}$ .

In the present investigation we studied synthesis and destruction of fatty acids in the animal organism on a carbohydrate diet. In mice, on a diet of whole wheat bread, the heavy water content of the body fluids was increased artificially to a definite level. It was found that the fatty acids of the animals took up stable deuterium<sup>4</sup> in a very short time. A maximum was reached after 6 to 8 days. Apparently an equilibrium between the deuterium content of the fatty acids and the body fluids was reached. The proportion of deuterium to protium in the total fat was about one-seventh of that in the body fluids at the equilibrium point.

In the course of our experiments the weight of the mice, and consequently the amount of body fat, remained constant. If then, the uptake of non-exchangeable deuterium by the fatty acids represents a synthesis, there must also have been a simultaneous destruction of fatty acids. After 6 days nearly all the original

<sup>3</sup> For a discussion of stable and labile deuterium in organic molecules see Paper I of this series (1).

<sup>4</sup> In all of our experiments we have been concerned only with stable deuterium, as only this form is of significance for our problem. The manner of preparation and the method of isolation of organic substances in our experiments (heating fatty acids in alkaline solution, etc.) effectively remove all labile deuterium.

fatty acids have been replaced by newly formed ones. This is clearly shown in our experiments by the constant deuterium content of the fatty acids after the 6th day. A continuous and rapid turnover of the fatty acids exists in the living organism.

In order to investigate more directly the breakdown of the fatty acids of animals on the same diet, we performed the following experiments: A large group of mice was fed for 5 days on a diet of whole wheat bread and 10 per cent of fat containing 5.1 atoms per cent of deuterium.<sup>5</sup> The animals, as shown in our previous studies (3), deposited part of this fat in their fat tissue. The depots were labeled by this procedure. Some of the animals were killed as controls after the 5th day, and their fatty acids analyzed for deuterium. The remainder were put on a diet of whole wheat bread. Groups of these mice were killed 2, 4, and 6 days later. The deuterium content of the fatty acids of these animals fell very rapidly and after 6 days was only about one-seventh of the starting value. The increase in the deuterium content of the fatty acids in our first experiment proceeds at about the same rate as the fall of the deuterium content in the second experiment. This must indeed be so, for in both cases we are measuring the same process: the rate of synthesis and simultaneous destruction on a carbohydrate diet.

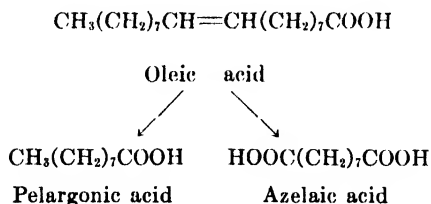
The method is so new and the results were so unexpected that a number of other experiments had to be devised to prove that we were actually measuring the rate of synthesis and destruction and not some other process. In the preceding communication (5) it was shown that saturated fatty acids can be desaturated in the animal organism. It is possible that hydrogenation of unsaturated fatty acids can also take place. It was necessary to determine, therefore, whether our finding actually represented synthesis and destruction or repeated hydrogenation and dehydrogenation. Hydrogenation proceeding in a medium of heavy water must lead to the introduction of deuterium atoms into the fat, while desaturation of a deuterium-containing fatty acid may lead to a loss of this isotope.

It is highly improbable that desaturation can account for the disappearance of deuterium from the fatty acids as observed in

<sup>5</sup> Atom per cent deuterium = per cent deuterium atoms in the total hydrogen atoms of the water or organic compounds.

the second experiment in which the mice were fed with fatty acids obtained by partial hydrogenation of linseed oil. There exists good evidence that the animal organism is not capable of forming linoleic or linolenic acid (9), the main constituents of linseed oil. The animal could, therefore, not desaturate at those positions where the deuterium atoms had been introduced in the acids of linseed oil.

We can show that hydrogenation is not responsible for the appearance of deuterium in the fatty acids in the first experiment. No fatty acids with double bonds between the carboxyl group and the 9th carbon atom are known in mammals. In all of the higher fatty acids of the animal organism this part of the molecule is saturated. Deuterium could not have entered this part of the molecule by biological hydrogenation of the fatty acids already present. We were, however, able to show that this part of the molecule of the unsaturated acids does contain deuterium. The unsaturated fatty acids were isolated from the fat of these animals according to the method previously described (5). The acids contained 0.12 atom per cent of deuterium and had an iodine number of 101, indicating that they consisted mainly of oleic acid. By ozonization followed by oxidation with chromic acid the double bonds were split and a mixture of mono- and dicarboxylic acids was obtained. Azelaic acid, derived from the part of the molecule between the carboxyl group and the double bond  $\Delta_{9,10}$ , was isolated in pure form from the mixture.



It contained 0.10 atom per cent of deuterium, a concentration about the same as that of the total unsaturated fatty acid fraction. Both halves of the unsaturated fatty acid molecules synthesized by the mice must therefore have contained approximately the same proportion of deuterium, and since the half forming azelaic acid could not have been involved in hydrogenation and dehydrogena-

tion, it follows that such processes cannot have been responsible for our results.

Another possibility is that an enzyme exists having the ability to labilize hydrogen atoms bound to carbon. An equilibrium between the hydrogen of the fatty acid chains and the hydrogen of the body fluids would result from the action of such a hypothetical enzyme, and, according to the relative concentrations of deuterium, it would either enter the fatty acid molecule or be given up to the water of the environment.

The living mammalian organism is not a simple subject for determining whether such an enzyme exists. These animals ingest food and convert its constituents continuously into other substances. A large number of such reactions, which have been the object of this investigation, can lead to the introduction of deuterium into the molecules. Most suitable for such experiments are organisms which ingest no food, and live for extended periods of time on the substances already present. We have utilized the developing hen's egg. The egg during development derives most of its energy from the fatty acid, the total amount of which decreases gradually (10). A physiological salt solution made up with heavy water was injected into the egg under sterile conditions on the 1st day of development, which proceeded then in a medium of 0.5 to 1.0 per cent heavy water. The embryos developed normally and no difference could be observed between the control and injected eggs. After 20 days the chicks had developed almost completely. If an enzyme with the ability to labilize hydrogen atoms of the fatty acid chains had been present, fatty acids isolated from the chicks should have contained appreciable amounts of stable deuterium. This was not the case. The concentration of deuterium was not increased in the fatty acids of the six animals which were analyzed. The presence of a hydrogen-labilizing enzyme in the developing chick can therefore be excluded. Developing chicks, as far as is known, contain all enzymes which are found in adult animals. On the basis of these experiments we feel justified in postulating that such an enzyme also does not exist in mice.

#### EXPERIMENTAL

*Experiment A. Synthesis of Fatty Acids in Mice on Carbohydrate-Rich Diet*—If the drinking water of an animal is replaced by a



dilute solution of heavy water, the concentration of  $D_2O$  in all of the body fluids will shortly become approximately constant. The tissue cells are unable to concentrate or dilute  $D_2O$  during the process of secretion and excretion. When equilibrium has been reached, the concentration of deuterium in the body fluids will be less than that of the drinking water, since ordinary water is formed in the combustion of food and is continuously being added to the body water. In preliminary experiments when mice on a bread diet were given water containing 2.35 atoms per cent of deuterium to drink, the concentration of deuterium in the body fluids became constant at approximately 1.5 per cent after a few days. As it is necessary, in studying the speed of synthetic processes quantitatively, that the concentration of  $D_2O$  in the body fluids be con-

TABLE I  
*Deuterium Content in Body Fluids and Fatty Acids of Mice Given Heavy Water*

Experiment No.	Duration of experiment	No. of animals	Combined weight of animals	Deuterium in body fluids	Deuterium in fatty acids	Hydrogen of fatty acids derived from body fluids
	days		gm.	atom per cent	atom per cent	per cent
1	3	4	69	1.21	0.11	9.1
2	4	10	185	1.09	0.08	7.2
3	6	4	68	1.43	0.21	14.6
4	9	4	70	1.50	0.20	13.3
5	19	3	53	1.51	0.22	14.5

stant throughout the experiment, we started our experiments by injecting subcutaneously a sufficient amount of 98 per cent  $D_2O$  to raise it to approximately 1.5 per cent at once.<sup>6</sup> This level was maintained thereafter by giving the mice 2.35 per cent heavy water to drink. Thus from the moment of injection all synthetic processes took place in a medium of approximately 1.5 per cent heavy water.

The analysis of the body fluids and the organic materials was carried out by a slight modification of the method previously described.<sup>7</sup> The results are given in Table I.

<sup>6</sup> The water content of the mice was considered to be approximately two-thirds of their total weight.

<sup>7</sup> The changes will be described in a forthcoming paper.

It is evident that the amount of deuterium in the fatty acids had reached a maximum after 6 days, since the values after 6, 9, and 19 days were the same within physiological and analytical limits of error. As the concentration of heavy water in the body fluids was also constant, a state of equilibrium between fatty acids and body fluids must have been established in this short period. In other words, all fatty acid molecules, which were susceptible to replacement, had been replaced by newly synthesized molecules. The "half-lifetime" of these molecules is less than 3 days, and the process appears to be surprisingly rapid.

In the first two experiments the concentration of heavy water in the body fluids was somewhat lower than in the others. The reason for the larger variation in the first days of the experiments is probably due to individual variations of water content of the mice, but whatever the cause, in these short experiments, synthesis proceeded in media containing 1.21 and 1.09 per cent  $D_2O$  instead of 1.5 per cent. In order to compare the results it is necessary, therefore, to calculate the ratio

$$\frac{\text{Atom per cent deuterium in fatty acids}}{\text{Atom per cent deuterium in body fluids}} \times 100$$

for each experiment. The results (last column of Table I) show the proportion of the total hydrogen of the fatty acid mixture originating in the body fluids. For example, if the atom per cent of deuterium in the fatty acids was one-tenth the atom per cent of deuterium in the body fluids, one-tenth of all the hydrogen atoms of the fatty acids must have come from the body fluids, assuming that the rates of reaction of protium and deuterium are the same.<sup>8</sup>

<sup>8</sup> In order to calculate the number of hydrogen atoms derived from the water one must know the value of  $\alpha$ , which is the ratio of the rates of reaction of protium to deuterium in these processes. Reitz and Bonhoeffer (8), who grew algae in high concentrations of heavy water (12 to 80 per cent) found  $\alpha$  to be about 2.5. If  $\alpha$  had this value in our experiments, then the number of such atoms would be increased by a factor of 2.5. Inasmuch as the natural compounds have approximately the same isotopic ratio as ordinary water (11), we are at a loss to understand the results of Reitz and Bonhoeffer. Although the value of  $\alpha$  has no direct bearing on the general physiological conclusions which we are drawing from our experiments, we are at present investigating the value by two independent methods.

Our results show that apparently an equilibrium is reached when an average of 13 to 14 per cent of all the hydrogen atoms of the fatty acids had been replaced.

*Experiment B. Destruction of Fatty Acids in Mice on Carbohydrate Diet*—Twenty-four male mice of the same weight were fed for 5 days on a diet consisting of 20 per cent fat and 80 per cent whole wheat bread. The fat was prepared by partial hydrogenation of linseed oil with deuterium as previously described (3). It contained 5.1 atoms per cent of deuterium. At the end of the 5 day period fifteen mice were killed as controls and divided arbi-

TABLE II  
*Deuterium Content of Fatty Acids of Mice after Discontinuation of Fat Feeding*

	Experiment No.	Days on fat-free diet	Combined weight of animals	Deuterium in fatty acids
			gm	atom per cent
Controls	1	0	57	0 97
	2	0	59	1 06
	3	0	59	0 72
	4	0	57	1 00
	5	0	65	1 05
Average . . . . .				0 96
Experimental mice	6	2	56	0 63
	7	4	57	0 35
	8	6	65	0 13

trarily into five groups of three animals each. The total fatty acids of each group were isolated and analyzed for stable deuterium. The results are consistent among themselves, considering the expected physiological variations, and average 0.96 atom per cent. The remaining nine mice were continued on a diet of bread alone. After 2, 4, and 6 days groups of three animals were killed and analyzed as in the case of the controls. The results (Table II) show that deuterium disappeared rapidly. After 6 days only 10 to 15 per cent of the original deuterium-containing fat remained. The "half-lifetime" in this experiment is also less than 3 days, and apparently the breakdown of fatty acids pro-

ceeded at about the same rate as the synthesis in the first series of experiments.

The parallelism of the rate of synthesis and destruction can be seen in Fig. 1. In Curve I we plot as a function of time the amount of deuterium in the fatty acids relative to the maximum value which they attained after 19 days, corrected for variations in the deuterium content of the body fluids. For example, the per cent synthesis on the 3rd day is given by

$$\text{Per cent synthesis} = \frac{0.11}{0.22} \times \frac{1.51}{1.21} \times 100 = 63$$

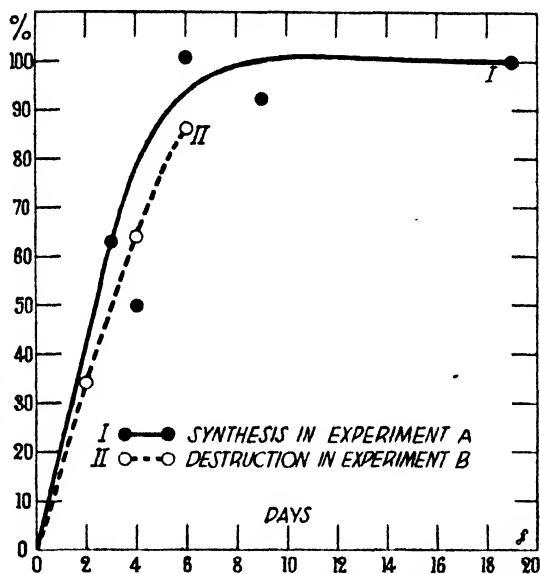


FIG. 1. Synthesis and destruction of fatty acids in mice

In Curve II we similarly plot the disappearance of deuterium in the fatty acids relative to the initial value.

*Experiment C. Separation of Unsaturated Fatty Acids*—As the fatty acids from mice which had received 2.35 per cent  $D_2O$  for 6, 9, and 19 days (see Table I, Experiments 3 to 5) contained the same concentration of deuterium, the three fractions were combined to furnish sufficient material (7.9 gm. in all) for further

investigation. The saturated and unsaturated fatty acids were separated according to the procedure described in the last communication (5). The saturated acids were precipitated as lead salts and the small amount of deuterium-containing saturated acids which remained in the mother liquor was washed out by repeated additions of natural stearic and palmitic acids. The resulting loss of unsaturated acids was so small as to be without significance. The saturated acids had an iodine number of 7.3 and a deuterium content of 0.42 atom per cent. In accordance with the calculations in Table I it may be computed that 28 per cent of all the hydrogen atoms in these saturated acids originated in the body fluids. If one assumes the average number of stable hydrogen atoms in this fatty acid mixture (stearic and palmitic acids) to be 34, about 9 or 10 hydrogen atoms must have come from the body fluids.<sup>8</sup> The unsaturated acid fraction had an iodine number of 101.3 and a deuterium content of 0.12 atom per cent. Only 8 per cent of all the hydrogen atoms originated in the body fluids, which is to say that of all the hydrogen atoms in these acids about 3 per molecule came from the body fluids.

*Experiment D. Ozonization and Oxidation of Unsaturated Acids. Isolation of Azelaic Acid from Oxidation Mixture*—The mixture of aldehydes and acids resulting from the ozonization and subsequent hydrolysis of a sample of the above unsaturated acid fraction was oxidized with chromic acid, and the resulting azelaic acid was isolated.

An amount of ozone equivalent to 1.5 times the theoretical amount (calculated from the iodine number) was passed into a solution of 2.5 gm. of the unsaturated fatty acids (see Experiment C) in 100 cc. of acetic acid in  $1\frac{3}{4}$  hours; 20 cc. of water and 2 gm. of chromium trioxide were added, and the solution was allowed to stand overnight. After addition of methyl alcohol to reduce the excess chromium trioxide the greater part of the solvent was distilled off *in vacuo* and the residue was distributed between ether and water. The aqueous layer was extracted continuously for 48 hours with ether, and the two ethereal solutions were combined. Most of the ether was distilled off, and the residue was treated with a current of steam. Azelaic acid is not volatile with steam and remained in the residue which was extracted with ether and the ethereal solution taken to dryness. Most of the ether extract

was soluble in hot water. The hot aqueous solution was treated with charcoal; on cooling 0.562 gm. of long white needles was obtained. After the substance was recrystallized from water the melting point was 105°, and was not depressed on mixing with pure azelaic acid.

$C_9H_{16}O_4$ .	Calculated.	C 57.40,	H 8.59
	Found.	" 57.56, 57.64,	" 8.35, 8.20

It was found to contain 0.10 atom per cent of deuterium. A correction is necessary in calculating the amount of deuterium in the corresponding fragment of the fatty acids from the deuterium content of the azelaic acid. The 2 hydrogen atoms on the carboxyl groups may not be included because they are labile. The true deuterium content of the fragment is therefore increased by 5 per cent, and is very nearly the same as that found in the total unsaturated fatty acid fraction from which the azelaic acid was obtained.

*Experiment E. Development of Chick Embryos in Medium of Heavy Water*—A solution of 0.9 per cent sodium chloride in 98 per cent heavy water was injected into ten fresh, fertilized hen's eggs. The method of injection was based on the procedure of Tomita (12) and Needham (13). The pointed end of the egg was sponged off with an alcoholic iodine solution and a small hole was bored through the shell with a sterile dental drill. 0.35 cc. of the solution was injected into each of five eggs, while the remaining five received 0.50 cc. The hole was closed with sterile adhesive plaster. Freshly sterilized drills and syringes were used for each egg. The eggs were incubated at 39°. No infection developed in any case. All but two, which probably had not been fertilized, developed normally. Two of the developing eggs were analyzed on the 9th day of incubation; the remaining six eggs were analyzed on the 20th day, *i.e.* immediately before hatching. In the latter the chicks were fully developed, and one had already begun to open the shell.

The total content of the egg was cut up with scissors and transferred to a round bottom flask. A few cc. of water were distilled off *in vacuo* at room temperature and the deuterium content of this water was determined. In the eggs which had received 0.35 cc. of  $D_2O$  the concentration of deuterium in the egg fluid ranged

from 0.47 to 0.65 atom per cent, and in the eggs which had received 0.5 cc. the concentration ranged from 0.81 to 0.98 atom per cent. The development had proceeded in a medium containing these concentrations of  $D_2O$ . After distillation of the water samples, the fatty acids were isolated by methods previously described, and their content of stable deuterium was determined. In no case was the concentration higher than 0.02 atom per cent. Within the limits of error of our procedure the fatty acids had therefore taken up no deuterium.

#### DISCUSSION

None of the foregoing five series of experiments is conclusive by itself, but taken together they show definitely that in living mice fatty acids are continuously destroyed and replaced. We are convinced that this process, disclosed by the aid of deuterium, represents a continuous and surprisingly rapid turnover of fatty acids in the organism. The results show that fatty acids present at the beginning of the experiment had been replaced in about 6 days. We do not wish to conclude that the complete turnover will always be found to require 6 days. On the contrary, some biological variation may be expected. The time will undoubtedly depend upon the type of diet and the general experimental conditions, and may vary within wide limits. However, while there can be no doubt that the process is a general one in all animals, it is to be expected that it will proceed faster in mice than in larger mammals, in which metabolism proceeds more slowly. It would be of great interest to determine on what factors this turnover depends. Unfortunately it is not at present feasible to extend the experiments to large animals because of the expense of heavy water.

What we have measured is an average for the fats of the entire organism. It may well be that the "half-lifetime" of different fatty acids or of the fats of different cells or organs may differ among themselves. This question concerning the fat metabolism of individual organs will be a subject for future work. Since the depots constitute by far the greatest part of the body fats, the characteristics here reported probably most closely resemble those of the depot fats.

It is generally believed that the primary function of the fat

depots, aside from their heat-insulating action, is to store energy for times of undernourishment. This function has been compared to that of a storage cellar. According to this theory the principal constituents of the fat depots, the fatty acids, should remain the same when there is no change in body weight. Changes would be expected only in fasting or in times of excess ingestion of food. Our experiments show that this is not the case, and that the fatty acids have only a short lifetime in the organism even when the body weight remains constant and there is consequently no change in the total amount of the fat stores.

The most important question raised by our findings is, what are the precursors of the newly synthesized fatty acids? In our experiments the mice received a carbohydrate-rich and fat-poor diet, and it seems almost certain that the new fatty acids were synthesized from the carbohydrates of the diet. It is well recognized that fat may be formed from carbohydrate and stored as fatty acids under conditions of overnourishment. Our experiments show that carbohydrates are continuously transformed into fatty acids under normal dietary conditions.

In our opinion the explanation for this process is as follows: Mice, like almost all other animals, take food only at intervals; the absorbed constituents of the diet are not burned immediately but must be deposited for short periods. Part of the carbohydrate (and carbohydrate precursors) is deposited as glycogen and is always available for combustion directly in the form of carbohydrate. However, the amount of glycogen which may be stored in the organs is relatively small. Most of the absorbed carbohydrate is, therefore, immediately transformed into fatty acids. These are deposited in the fat depots and utilized for combustion in the postabsorptive periods. This mechanism offers an explanation for all of our findings. The mouse nourished on carbohydrate forms fatty acids continuously, while the developing hen's egg, which receives no food and lives throughout the entire period of development on substances already present, synthesizes no fatty acids.

The results show that the fat depots represent a much more active organ than has been thought hitherto. In a recent publication (3) it was shown that the fat of the diet, even if it constitutes only a small part of the total, is deposited to a large extent



in the fat tissues. It has now been found that a similar process takes place in the case of carbohydrate, of which a part also enters the fat depots. Instead of comparing the fat tissues to a cellar in which food is stored for times of emergency, it seems more correct to compare them to an ice box in which a part of the food is kept during the short intervals between meals. *The fat tissue can therefore be regarded as an energy buffer. During absorption it takes up in the form of fatty acids excess of food material not immediately used for the energy requirements. Conversely, during the postabsorptive period it supplies fatty acids to make up the energy deficit.*

In contrast to the adult mammal, the developing hen's egg forms no new fatty acids. Transformation of carbohydrate into fatty acids would not be expected, since the freshly laid egg contains only a small amount of glycogen. Furthermore, the absence of deuterium in the fatty acids at the end of development excludes the possibility that unsaturated fatty acids had been hydrogenated during development. If hydrogenation had occurred, there would have been an introduction of deuterium into the fatty acid molecules, since the reaction proceeded in a medium of heavy water. In our last communication (5) it was shown that mice are able to dehydrogenate saturated fatty acids. The present results with eggs have raised the question whether hydrogenation of unsaturated acids may occur in mammals. We are investigating this problem.

The results which we have presented raise other questions which can only be mentioned but not discussed in detail at this time. After equilibrium had been reached in the biological synthesis of fatty acids in Experiment A, 13 to 14 per cent of the hydrogen atoms of the acids had come from the body fluids. In separating the mixture into saturated and unsaturated acids (Experiment C) it was shown that the amount of deuterium was different in the two groups. The saturated acids had taken up 28 per cent and the unsaturated acids only 8 per cent of their hydrogen atoms from the body fluids. No explanation for this difference is yet apparent. It is possible that both fatty acid fractions are formed at equal rates and that the higher deuterium content of the saturated acids depends upon a secondary hydrogenation of the unsaturated acids. However, the explanation is

highly improbable because the difference between the two fractions is so great that a hydrogenation must be assumed of more double bonds than are present in the unsaturated fatty acids. This explanation also depends upon the assumption that animals are able to hydrogenate fatty acids, for which there is as yet no proof. It is much more probable that saturated acids are formed first and then partially dehydrogenated. We believe that the explanation of the differences observed will furnish an important insight into the mechanism of intermediary fat metabolism.

Knowledge of the positions of the deuterium atoms in the molecules of fatty acids formed in the animal body should furnish a key to the mechanism of synthesis. It should be possible to secure such information by means of stepwise chemical degradation.

#### SUMMARY

1. Deuterium can be used for the study of synthesis and destruction of organic molecules in the intact living organism. The rates of these reactions can be determined.

2. The synthesis of fatty acids in mice on a diet rich in carbohydrate has been followed by raising the deuterium content of the body fluids to 1.5 atoms per cent. The deuterium content of the fatty acids rose rapidly and attained a maximum value in 6 to 8 days.

3. The simultaneous destruction of fatty acids on the same diet was demonstrated in another experiment. Deuterium-containing fatty acids which had previously been deposited in the fat tissues disappeared with about the same speed.

4. Azelaic acid derived from the unsaturated acids synthesized by the mice which had been given heavy water had the same deuterium concentration as the total fatty acids. This finding proves that our results are not due to successive saturation and desaturation.

5. The fatty acids of hen's eggs developing in a medium of heavy water do not take up deuterium into their molecules. This rules out the possibility that there exists an enzyme which can catalyze the exchange of hydrogen atoms of these fatty acids with water. It furthermore indicates that in eggs there does not occur an appreciable amount of hydrogenation of unsaturated fatty acids.

6. The rapid turnover of the fatty acids in mice shows that there is a continuous conversion of carbohydrates to fatty acids under normal dietary conditions.

7. The rôle of the fat tissue as an energy buffer for the organism is discussed.

The authors are highly indebted to Mr. M. Graff for his valuable assistance in the course of these experiments.

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# THE IONIZATION CONSTANTS OF CALCIUM PROTEINATE DETERMINED BY THE SOLUBILITY OF CALCIUM CARBONATE

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The work to be reported in this paper deals, as does the recent one of McLean and Hastings (1), with the nature of the combination between calcium and proteins. Whereas in the preceding paper, the calcium ion concentration was estimated by a biological method, in the present paper, a physicochemical method was used.

## *Methods*

### *Theoretical Considerations*

The hypothesis to be investigated was, that the combination between calcium and protein in solution is a chemical one, and that the quantitative relation between calcium ions  $[Ca^{++}]$ , protein ions  $[Prot^-]$ , and the calcium salt of protein  $[CaProt]$  can be expressed by the mass law equation

$$(1) \quad \frac{[Ca^{++}] \times [Prot^-]}{[CaProt]} = K$$

or expressed logarithmically

$$(2) \quad -\log [Ca^{++}] - \log [Prot^-] + \log [CaProt] = -\log K = pK_{CaProt}$$

This relationship was first suggested by McLean and Hastings (1) and is discussed in detail in their paper. It was the purpose of the present investigation to test the application of Equation 1 to protein solutions, only chemical methods being used for the determination of the variables, and to extend the observations over a wider pH range than was possible when the frog heart was used for the determination of  $[Ca^{++}]$ .

## 398 Ionization Constants of Ca Proteinate

In the experiments to be reported in this paper, the  $[Ca^{++}]$  was estimated as follows: Solutions were brought into equilibrium with solid  $CaCO_3$  at a definite  $CO_2$  tension and at  $38^\circ$ . Under these conditions, one may apply the solubility product relationship

$$(3) \quad [Ca^{++}] \times [CO_3^{--}] = K'_{s.p.}$$

where  $[Ca^{++}]$  and  $[CO_3^{--}]$  are stoichiometric ion concentrations and  $K'_{s.p.}$  is the stoichiometric solubility product constant for the particular temperature and ionic strength of the solution used in the experiments. The values used for  $K'_{s.p.}$  were those reported by Hastings, Murray, and Sendroy (2).

TABLE I

*Factors for Conversion of Protein Concentrations (Expressed As Gm. per Kilo of  $H_2O$ ) into Divalent Protein Anions ( $Prot^{--} + CaP$ ) (Expressed As MM per Kilo of  $H_2O$ ) at Different pH Values*

pH	Casein	Globulin	Albumin
7.0	0.255	0.081	0.114
7.2	0.293	0.089	0.127
7.4	0.323	0.097	0.139
7.6	0.348	0.104	0.152
7.8	0.365	0.112	0.165
8.0	0.388	0.119	0.177

$[CO_3^{--}]$  was calculated in each solution from the total  $CO_2$  and pH by Equation 4.

$$(4) \quad [CO_3^{--}] = \frac{[total\ CO_2] K''_1 K'_2}{[H^+]^2 + [H^+] K''_1 + K''_1 K'_2}$$

where  $K''_1 = 7.41 \times 10^{-7}$ ;  $K'_2 = 1.62 \times 10^{-10}$ .

Since  $[CO_3^{--}]$  and the appropriate value of  $K'_{s.p.}$  for the solutions were known,  $[Ca^{++}]$  was calculated from Equation 5.

$$(5) \quad [Ca^{++}] = \frac{K'_{s.p.}}{[CO_3^{--}]}$$

The calcium combined with protein  $[CaProt]$  was calculated by subtracting the  $[Ca^{++}]$  from the total calcium concentration.

The protein concentration was multiplied by an appropriate factor to give the concentration of protein anions expressed as divalent ions (Table I). The value for  $CaProt$  was subtracted

from this figure and the remainder was designated [Prot<sup>-</sup>]. The factors for casein were calculated from the data of Pertzoff and Carpenter (3); those for albumin and globulin from the data of Van Slyke *et al.* (4).

Having thus obtained values for [Ca<sup>++</sup>], [Prot<sup>-</sup>], and [CaProt], we calculated values for pK<sub>CaProt</sub> (Equation 2).

### Experimental Procedure

*Preparations of Proteins*—The proteins used were prepared from fresh beef serum. The total serum globulins and serum albumin were separated by half saturation and complete saturation with ammonium sulfate respectively. The proteins were then electro-

TABLE II

*Length of Time Required for Equilibrium in Undersaturated Solutions of Casein*

Ex- peri- ment No	pH	[CO <sub>2</sub> ]	[CO <sub>2</sub> ]	[Ca <sup>++</sup> ]	Total [Ca]	[Ca- Prot]	[Prot <sup>-</sup> ]	Protein	pK	Time
		mm per kg H <sub>2</sub> O	mm per kg H <sub>2</sub> O	mm per kg H <sub>2</sub> O	mm per kg H <sub>2</sub> O	mm per kg H <sub>2</sub> O	mm per kg H <sub>2</sub> O	gm. per kg. H <sub>2</sub> O		hrs
1	7 36	19.23	0 067	0 59	1 01	0 42	9 43	31 80	1 88	1
2	7 34	19.44	0 065	0 62	1 10	0 49	9.37	31.80	1.93	1
3	7 56	18 33	0 105	0 38	1 08	0 70	9 95	31 80	2 27	2
4	7 63	18 41	0 123	0 32	1 06	0 75	14 21	43 37	2.22	5

dialyzed to remove the diffusible electrolytes. In certain experiments Pfanstiehl's preparation of casein was used.

*Preparation and Treatment of Solutions*—After dialysis solid NaCl was added to the protein in an amount calculated to give a final ionic strength of approximately 0.160. A standard solution of NaOH was then added in such quantity that the pH of the final solution would be approximately 7.4 and the bicarbonate concentration approximately 25 mm per liter in the presence of a CO<sub>2</sub> tension of 40 mm. Carbon dioxide was added to the solution until the desired pH was obtained. The solution containing protein, NaCl, and NaHCO<sub>3</sub> was then equilibrated with solid CaCO<sub>3</sub>. Equilibration was carried out in 50 cc. centrifuge bottles with constricted necks, in a water bath maintained at 38° and for a time sufficient to attain equilibrium (Table II).

After equilibration, the solutions were removed from the thermostat, immediately covered with paraffin oil, and centrifuged to remove the excess solid  $\text{CaCO}_3$ . The supernatant solution was pipetted off under oil and transferred to a wide mouthed test-tube containing oil. The solutions were analyzed for pH, total  $\text{CO}_2$ , total calcium, and protein. In the tables the results have been expressed as mM or gm. per kilo of water.

#### *Analytical Procedure*

*Determination of pH*—The method used for pH was the bicolor method described by Hastings and Sendroy (5). Under the conditions of these experiments, a correction factor for the effect of the proteins upon the color of the indicator must be introduced. This correction factor was determined as follows: (1) The pH of a protein solution was determined in the usual manner by adding 0.2 cc. of the protein solution to 4.0 cc. of adjusted phenol red-saline solution. (2) The colorimetric pH of a phosphate buffer, so chosen that it had approximately the same pH as the protein solution, was similarly determined. (3) Then the pH of the buffer, plus phenol red, plus 0.2 cc. of the protein solution was determined. Since the protein was in such low concentration that it could not affect the pH of the buffer, the true pH of this solution was taken as that of the buffer without protein. The difference between (2) and (3) was the correction to be added to the apparent pH of the protein solutions. It was found that 0.09 should be added to the apparent pH of albumin solutions and 0.05 to the apparent pH of globulin solutions used in our experiments.

*Determination of  $\text{CO}_2$* —Carbon dioxide was determined by the manometric method of Van Slyke and Neill (6).

*Determination of Calcium*—Calcium was determined by the method of Kramer and Tisdall (7). 5 cc. samples were used and determinations were made in triplicate.

*Determination of Protein*—Proteins were determined by the macro-Kjeldahl method with selenium as the catalyst.

#### *Results*

*Time Necessary to Reach Equilibrium*—Preliminary experiments to determine the time required to reach equilibrium with  $\text{CaCO}_3$  were made on solutions of casein. The criterion of the establishment

of an equilibrium condition was taken to be the attainment of a constant value for  $pK_{CaProt}$ . The results are shown in Table II. Equilibrium was apparently reached within 3 hours. It was decided as a precaution arbitrarily to double the minimum equilibration time in subsequent experiments. Unless otherwise stated, the experiments to be reported have been equilibrated from 5 to 6 hours.

The effect of varying the pH of casein solutions from 7.21 to 7.69 on the value of  $pK_{CaProt}$  is shown in Table III. The results show that the value of  $pK$  remained constant over the pH range studied.

*Effect of Adding Excess  $Ca^{++}$  Initially to the System  $NaCl$ ,  $NaHCO_3$ , Protein (Liquid Phase) and  $CaCO_3$  (Solid Phase)*—In view of the tenacity with which certain systems supersaturated with respect to  $CaCO_3$  hold the excess calcium carbonate in solution (8), an attempt was made to reach equilibrium in the protein solutions from an initially supersaturated state. Table IV gives the results of these experiments on solutions of casein. It will be seen that calcium did not precipitate from the solution even after equilibration for 13 hours. In Column 10 are given values of  $Ca^{++}$  concentrations calculated on the basis of Equation 5 and  $pK$  2.23. In Column 9 are given the concentrations of calcium ions which would have been expected had the solution been in equilibrium with solid  $CaCO_3$ . The fact that the former far exceeds the latter indicates that the solution was greatly supersaturated with respect to  $CaCO_3$  and remained so for 13 hours. This experiment demonstrated that it was not possible to attain equilibrium within a reasonable time in supersaturated solutions.

*Effect of Varying Globulin Concentration*—The results of equilibrating solutions of serum globulin varying in concentration from 19 to 75 gm. per kilo of water with solid  $CaCO_3$  are shown in Table V. It will be seen that a fairly constant value for  $pK_{CaProt}$  (average 2.32) was obtained, indicating that within the range of protein concentration studied, the combination between calcium and globulin may be adequately described by Equation 2.

*Effect of Varying pH of Globulin Solutions*—The results of varying the pH from 7.04 to 7.91 on the value of  $pK_{CaProt}$  are shown in Table VI. No variation of  $pK$  outside the limits of experimental error was observed and the average value for  $pK$  in these





[illegible]

experiments (2.38) agrees quite well with the value obtained in the experiments of Table VI. It should be noted that the globulin used in these experiments was a different preparation from that used in the previous experiments.

In these experiments the solid  $\text{CaCO}_3$  was removed by centrifugation from the solutions after equilibration for 2 hours and replaced with fresh  $\text{CaCO}_3$ . The solutions were then equilibrated again for 6 hours. This was done to eliminate the possibility that the solid  $\text{CaCO}_3$  had become coated with protein and consequently incapable of dissolving in the solution.

*Effect of Varying Albumin Concentration*—In Table VII are presented the results obtained with serum albumin. The value of  $\text{pK}_{\text{CaProt}}$  (average 2.20) is slightly less than that obtained for serum globulin. It will be noted that the value is constant from 17 to 70 gm. of albumin per liter.

*Effect of Varying pH of Albumin Solutions*—The results of varying the pH of serum albumin solutions from 7.32 to 7.82 are given in Table VIII. It will be observed that the value for  $\text{pK}_{\text{CaProt}}$  obtained is constant within the limits of experimental error, and that the average value obtained for  $\text{pK}_{\text{CaProt}}$  agrees with that shown in Table VII.

#### DISCUSSION

In general, it may be stated that the observations made in this paper are consistent with the hypothesis that calcium combines with proteins to form salts which are partially ionized into  $\text{Ca}^{++}$  and protein ions. Further, that an equilibrium exists between these ions and the un-ionized calcium-protein salt, which may be quantitatively expressed by the mass law Equation 1.

As was pointed out by McLean and Hastings (1), the simple relation,  $(\text{Ca}^{++} \times \text{protein})/\text{CaProt}$ , yields a figure which is approximately constant for serum. Another relation which would fit equally well is  $(\text{Ca}^{++} \times \text{Prot}^-)/\text{CaProt}^+$ . At the present time, it is not possible to choose among these on the basis of the experimental data. However, because the result of combination between  $\text{Ca}^{++}$  and citrate ions appears to be a salt in which calcium is combined with two carboxyl groups, the relation analogous to this (Equation 2) is adopted here.

A comparison of the mean values of  $\text{pK}_{\text{CaProt}}$  found in the

present experiments with those found by McLean and Hastings, with the frog heart method, is shown in Table IX. In the case of globulin, the present value is 0.12 higher and in the case of albumin 0.18 higher than those found by the previous authors. Whether or not this is to be attributed to the fact that the present work was carried out at 38°, whereas the previous work was carried out at room temperature, is uncertain. The values of  $pK_{CaProt}$  reported in this paper would lead to a value for normal serum of 2.29 compared with the previously reported value of 2.22. With the former instead of the latter value, this would make a difference in the calculated  $Ca^{++}$  concentration of serum of only 0.1 mM per liter, an amount not outside the limit of error of the analyses involved.

TABLE IX  
Mean Values of  $pK_{CaProt}$

Protein	$pK_{CaProt}$	
	McLean and Hastings	Weir and Hastings
Globulin.....	2.24	2.36
Albumin.....	2.03	2.21
Serum.....	2.22	2.29*

\* Calculated for serum with an albumin to globulin ratio of 1.5.

#### SUMMARY

1. Determinations of the solubility of calcium carbonate in solutions of casein, serum albumin, and serum globulin have been made at 38° for different pH values and protein concentrations.

2. From these data, the ionization constants of the calcium salts of these proteins have been calculated and found to have the following values: casein,  $pK_{CaProt} = 2.23$ ; globulin,  $pK_{CaProt} = 2.32$ ; albumin,  $pK_{CaProt} = 2.20$ .

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## LONG SPACINGS IN MACROMOLECULAR SOLIDS

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PLATES 4 AND 5

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Current ideas upon the structure of solids with large molecules are commonly based on deductions from x-ray pictures of cellulose, rubber, and similar polymerized organic substances. According to these views (1) the crystal is not built up of an association of large molecules all of equal shape and weight. Instead it consists of many of the unassociated molecules bound together through primary valencies into chains which extend parallel to the fiber axis. Sometimes it has been assumed that the atoms in these chains have the three dimensional regularity of arrangement characteristic of true crystals; others seem to require regularity only in the direction of fibering. This picture of structure has been applied to a wide variety of substances: to synthetic products such as the polyoxymethylenes, to rubber, to cellulose and other polysaccharides, and to the fibrous proteins like keratin. It is in accord with many of the observed properties of organic polymers and very probably contains much truth. The objection that must be raised against it is this, that the x-ray data from which it has been developed are not adequate to show whether it is true or false.

x-Ray diffraction studies can be made that will indicate whether this simple chain structure is essentially correct or whether the fiber is more properly to be considered as a regular aggregate of large discrete molecules. This decision must depend on the existence and the character of large spacing reflections that may be present in the diffraction diagram. If a crystal is composed of large molecules, then it must exhibit repetition distances of the same order of magnitude as the molecular diameter; on the other

hand there is no reason why a chain-like structure need have spacings much larger than the unit which is polymerized. If cellulose, for example, is an assemblage of discrete molecules all equal in size, like the simple organic crystals, its x-ray pattern should contain large spacing reflections corresponding to a presumably high molecular weight. If, however, it has a chain-like polymeric structure, these large spacings might be expected to be absent.

Most diffraction studies of macromolecular solids, including all the earlier ones, are incapable of showing whether or not these large spacings really exist. Long spacings have correspondingly small angles of reflection; hence in the usual diffraction photograph they lie crowded together either in or directly adjacent to the central undeviated beam. In order to observe and measure them with any degree of accuracy several steps must be taken: (1) cameras of increased radius and (2) smaller slits giving more restricted x-ray beams must be employed; (3) the x-ray wave-length must be made as long as feasible; (4) extraneous x-ray scattering must be reduced to a minimum by careful design and accurate construction of the camera and by the use of special gaseous atmospheres or a vacuum during photography. Perhaps the greatest difficulty in recording long spacings lies in producing the exceptionally intense x-ray beams that are necessary; every increase in camera radius and every decrease in slit size inevitably results in a pronounced weakening of the entire diffraction pattern.

Since it is obvious that no interpretation of the structure of any macromolecular solid can be satisfactory unless it is based on a knowledge of the complete diffraction diagram, we have gradually been developing apparatus to record any large spacings that might exist. With the tube and cameras recently described (2) it is feasible to make good measurements of all spacings smaller than about 130 Å. on either fiber or powder diagrams. Photographs thus obtained from typical macromolecular organic substances are discussed in the following paragraphs. For this discussion rubber has been chosen as a representative hydrocarbon polymer, cellulose and chitin as typical polysaccharides, chymotrypsinogen as a soluble crystalline protein, and collagen, keratin, and silk fibroin as insoluble crystalline proteins.

Very large spacings are already known from a number of these substances. Thus several soluble proteins (3) have recently been

photographed under conditions capable of bringing out large spacings. They all, when assuredly undecomposed, have shown true crystalline diffraction patterns with many reflections of spacings up to 50 Å. or more. Spacings of the order of 25 Å. have been described from keratin (4) and longer spacings (70 to 80 Å.) have been found in tendon (5) and in nerve (6). It has also been said (7) that very long spacings are present in keratin, rubber, and perhaps in cellulose. Some of these last statements are in serious conflict with the photographs treated in the present paper, but no data have been published in support of them and it is therefore impossible to state with certainty the source of the discrepancy.

Photographs of chymotrypsinogen,<sup>1</sup> tendon, and keratin are reproduced in Figs. 1 to 4; pictures of chitin, cellulose, purified silk fibroin, and stretched rubber in Figs. 5 to 8. Some spacing measurements upon these patterns are recorded in Tables I and II. When the scattering at small angles, corresponding to large spacings, is considered, these diagrams fall into three groups represented by (1) rubber, (2) collagen, (3) cellulose. In diffraction photographs made under the same experimental conditions rubber shows no inner reflections (Fig. 8) and collagen gives many strong reflections of large spacing (Fig. 1). The cellulose pattern (Fig. 6) seemingly contains no sharp reflections at small angles, but it always displays a band of intense unresolved scattering that runs normal to the fiber axis and reaches to the smallest observable diffraction angles. The meaning of this band is not yet clear; it is conceivable that subsequent work at higher resolving powers might show individual reflections within it, but such a result now seems unlikely.

Of the various macromolecular solids with which we have worked, rubber itself is the only one that has not as yet shown any characteristic diffraction at small angles. The simplicity of its diagram strongly suggests that the unit cell of rubber hydrocarbon really is small and that the prolonged chain-like structure commonly proposed may well be a proper description of the kind of molecular arrangement it possesses. The details of this arrangement are of course not known; atomic positions can only be established after a far more exhaustive analysis than has yet been made

<sup>1</sup> This chymotrypsinogen was made and several times recrystallized according to the procedures of Kunitz and Northrop (8).



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TABLE I

*Reflections Measured on Photographs of Insoluble Crystalline Proteins*

	Tail tendon (kangaroo), Fig. 1		Feather keratin (fowl), Fig. 2		Quill keratin (Canadian porcupine), Fig. 3	
	Intensity	Spacing	Intensity	Spacing	Intensity	Spacing
		Å.		Å.		Å.
Reflections on merid- ian (fiber axis ver- tical)	Strong	2 91	Medium	3 07	Strong	5 17
	Faint	4 03	Faint	3 22	Faint	10.5
	"	7 21	"	3 52	"	13 2
	Very faint	21.6	"	3 95	"	19 8
	" "	24.1	Medium	4 37	"	27 7
	Faint	26.9	Strong	4 90	Medium	66 3
	Medium	33 6	"	6.20		
	Strong	54 6	Faint	9 08		
	"	70 1	"	17 2		
	"	103	Strong	23 1		
Reflections on equator	Medium	5 42	Strong	4 68	Halo	4.6
	Strong	10 9	(diffuse)		Medium	7 61
	Faint	19 9	Medium	8 56	(diffuse)	
	Very faint	30 0	"	11 0	Strong	9 35
	Faint	47 6	Faint	17 1	(diffuse)	
			Strong	33 3	Medium	26 4
			Faint	51 0	Faint	41 9
			Medium	81 8	Strong	73 8
			"	115	"	112

TABLE II

*Some Powder Reflections from Chymotrypsinogen (Fig. 4)*

Intensity		Intensity	
Spacing		Spacing	
Å.		Å.	
Medium	3.60	Very faint	6 88
Faint	3 87	Faint	7 74
Very faint	4 64	Medium	8.62
Medium	4 98	Strong (broad)	10.13
Faint	5 44	Medium	13 42
"	6.06	Very faint (broad)	17.4
		Strong	24.9

of the positions and intensities of observed diffraction spots. The only known evidence against such a simple structure for rubber is the statement (7) that gel rubber (an ether-insoluble fraction) gives a 58 Å. spacing. If this is true and if the spacing should be due to rubber, then the problems of its structure would immediately become far more complex.

The cellulose pattern seems to be typical of polysaccharides. Absence of large spacings along the direction of fibering is certainly in accord with the view that these structures, too, are chain-like and have simple unit cells, "but any satisfactory picture of their structure must explain the unresolved scattering at small angles. Silk fibroin is the only non-polysaccharide known to give a similar scattering. This protein, when purified, shows an inferior degree of crystallization, however (Fig. 7), and from the standpoint of our present inadequate knowledge it is conceivable that this imperfection of arrangement might play a part in producing such diffraction.

Whether or not the well crystallized fibrous proteins eventually prove to have a similar chain-like basis, consideration of the patterns they give does not make such a structure inevitable. The unit cells of both collagen and keratin are certainly very large. Equatorial spacings of the order of 50 Å. in tendon necessarily mean that the unit cannot be merely an elongated prism of small cross-sectional area; in keratin these spacings normal to the fiber axis exceed 100 Å. The fiber spacing in tendon is probably about 330 Å. This large repetition distance would be obtained if connective tissue were built up of sufficiently big molecules, all alike. Conceivably it also could be obtained from a structure composed of chains of indefinite length, provided these chains were on the average many times 300 Å. in length and were built up of a succession of amino acid residues which repeated itself exactly every 300 Å. There are obviously disadvantages to such a molecular model for tendon and it is even more difficult to reconcile it with the 100 Ångström unit spacings of keratin. Nevertheless a conclusive discrimination between these two types of structure should be based on more data than we now possess. Help in choosing between chains and discrete molecules could be obtained through studies of molecular sedimentation. Such measurements on tendon have already been undertaken in this laboratory and the

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diffraction measurements are being extended to establish the longest spacings present.

### SUMMARY

x-Ray diffraction photographs capable of registering very large spacings (up to about 130 Å.) have been made from a number of macromolecular solids. The patterns thus obtained fall into three groups: (a) rubber-like patterns, which show no large spacings; (b) polysaccharide patterns, which show no separate large spacing reflections but have an apparently continuous band of scattering reaching to the shortest measurable angles; (c) protein patterns, which show numerous sharply defined large spacings.

Substances giving pattern (a) have small units which very probably build up the commonly accepted chain-like structures. The crystallographic unit cells for (b) likewise may be small. All the well crystallized proteins thus far examined, except fibroin, belong to group (c) and have large units with volumes of the order of magnitude of whole protein molecules. While this does not exclude the possibility of proteins like keratin and collagen also being chain-like in their structure, it shows that for the present they can be equally well viewed as ordinary molecular crystals built up by the regular arrangement of very large molecules.

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## EXPLANATION OF PLATES

All the photographs are arranged so that the traces of the fiber axes are vertical, *i.e.* run from top to bottom of the page.

## PLATE 4

FIG. 1, *a*. A diffraction pattern of the tail tendon of the kangaroo made with the pinhole x-ray beam normal to the axis of the tendon (the fiber axis). Tendon to film distance (camera radius), 3.0 cm. Cu radiation.

FIG. 1, *b*. The pattern of a similar preparation photographed in a camera of greater radius (7.3 cm.). Corresponding reflections are readily identified. The long spacings along the trace of the fiber axis commence to be visible. Cu radiation.

FIG. 1, *c*. The long spacing pattern of a similar preparation photographed with chromium radiation in a camera of 15.1 cm. radius. All of the resolved reflections of Fig. 1, *b* are off the picture. Pinhole diameter, 0.3 mm. Cr radiation.

FIG. 2, *a*. A diffraction pattern made by x-rays passing through the quill of an unstretched chicken feather. Camera radius, 3.0 cm. Cu radiation.

FIG. 2, *b*. The long spacing pattern of a similar chicken quill made with a 15.1 cm. radius camera. The strong vertical reflections of this picture just show in Fig. 2, *a* immediately above and below the central stop. Cr radiation.

FIG. 3, *a*. A portion of a diffraction pattern through an unstretched quill of the Canadian porcupine. (We are indebted to Captain R. Cheyne-Stout, Director of Menageries, New York, for supplying this and many other preparations.) Camera radius, 3.0 cm. Cu radiation.

FIG. 3, *b*. The long spacing pattern of a similar preparation made with a camera of 15.1 cm. radius. The large diffuse reflection at the right is to be identified with one of the two bright horizontal spots of Fig. 3, *a*. Cr radiation.

FIG. 4. A pattern of chymotrypsinogen made in a 7.4 cm. radius camera. Numerous powder rings can be seen; the original photograph shows that they extend down to the central stop. Cu radiation.

## PLATE 5

FIG. 5, *a*. The diffraction pattern from lobster chitin. Camera radius, 3.0 cm. Cu radiation.

FIG. 5, *b*. The pattern of a similar preparation made with a 15.1 cm. radius camera. Both of the strong equatorial reflections of Fig. 5, *a* can be seen. Cr radiation.

FIG. 6, *a*. The diffraction pattern from rayon fibers (cellulose). Camera radius, 3.0 cm. Cu radiation.

FIG. 6, *b*. The pattern of a similar preparation made with a 15.1 cm. radius camera. All the strong equatorial reflections, as well as the small angle scattering, are visible. Cr radiation.

FIG. 7, *a*. The diffraction pattern of purified silk fibroin. This sample

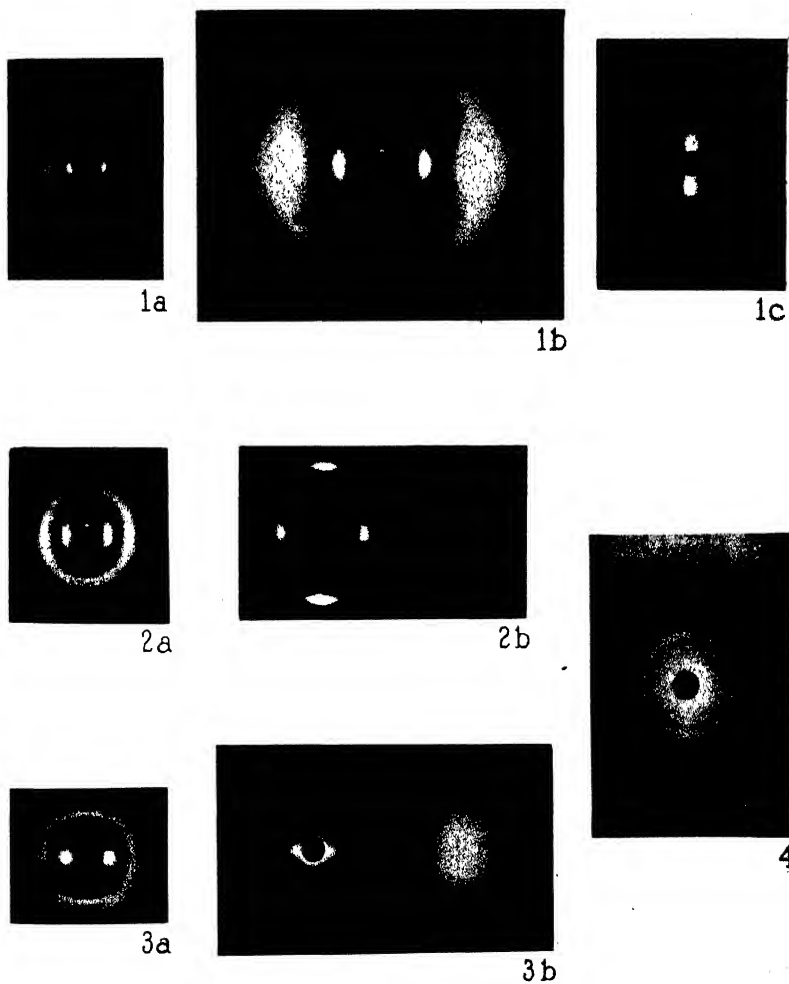
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supplied by Dr. M. Bergmann was degummed silk which had been repeatedly boiled with water. Its relatively poor orientation is evident. Camera radius, 3.0 cm. Cu radiation.

FIG. 7, *b*. The photograph of a similar preparation with a 15.1 cm. radius camera. The general scattering at small angles, most of it concentrated along the equator, is evident. Cr radiation.

FIG. 8, *a*. A portion of the diffraction pattern of stretched rubber dam. Camera radius, 3.0 cm. Cu radiation.

FIG. 8, *b*. A photograph of a similar preparation with a 15.1 cm. radius camera. Cr radiation.







a



5

b



a



6

b

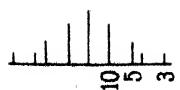
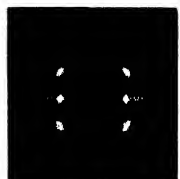


a



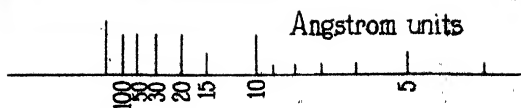
7

b



8

b



Angstrom units





## AN EXAMINATION OF THE SULLIVAN COLORIMETRIC TEST FOR GUANIDINE

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The detection of free guanidine by the colorimetric method developed by Sullivan (1) is of interest not only in clinical studies connected with muscular dystrophies, but also to all workers in the guanidine field. While the reaction is reported to be highly specific for guanidine, and is "not given by methyl guanidine or any other substituted guanidine" (2), a positive result (a red color in the solution under test following the addition of concentrated nitric acid) was obtained by Sullivan with guanidineglyoxylic acid and guanidineoxalic acid (1). Ammonia, indole, and methylamine also gave positive reactions.

Having available in this laboratory a number of new mono- and diguanidine derivatives, we decided to extend the Sullivan test to these compounds with the idea of furnishing additional data on the specificity of this reaction.

Two series of tests were made on each guanidine; one, with the concentration of guanidine derivative calculated on the basis of the amount of substituted guanidine salt equivalent to 0.5 mg. of free substituted guanidine base per cc., and the second, calculated on the basis of the amount of substituted guanidine salt equivalent to 0.5 mg. of free unsubstituted guanidine base per cc. The 1,2-naphthoquinone-4-sodium sulfonate used was prepared by the method of Folin (3). No mixtures of guanidines or of guanidines with other types were investigated. In every test a direct comparison was made with guanidine hydrochloride. The reaction was carried out six times on those guanidines with which positive results were obtained, while the negative tests were carried out in duplicate.

The following guanidines gave a negative result (yellow color

instead of red after addition of nitric acid): cyclohexylguanidine hydrochloride, hexahydrobenzylguanidine sulfate,  $\beta$ -cyclohexylethylguanidine sulfate,  $\alpha, \alpha'$ -diguanidodi( $\beta$ -thiopropionic acid), 1,6-diguanido-*n*-hexane sulfate, benzylbiguanide hydrochloride, phenylaminoguanidine hydrochloride,  $\alpha, \alpha'$ -diguanidodi( $\beta$ -thiopropionic acid) dihydrochloride, anhydro- $\alpha, \alpha'$ -diguanidodi( $\beta$ -thiopropionic acid) dihydrochloride,  $\alpha, \alpha$ -phenylbenzylguanidine hydrochloride,  $\alpha, \alpha$ -dibenzylguanidine hydrochloride,  $\alpha, \gamma$ -dibenzylguanidine hydrochloride,  $\alpha, \beta, \gamma$ -tribenzylguanidine trihydrochloride, *m*-tolylguanidine sulfate, *p*-tolylguanidine hydrochloride,  $\alpha, \alpha$ -phenylmethylguanidine hydrochloride, *p*-bromophenylguanidine hydrochloride, *p*-iodophenylguanidine hydrochloride,  $\beta$ -phenyl- $\alpha$ -guanidopropionic acid, *n*-hexylguanidine sulfate, benzylguanidine sulfate, (*dl*) $\alpha$ -phenylethylguanidine sulfate,  $\beta$ -phenylethylguanidine sulfate, (*dl*) $\beta$ -phenylisopropylguanidine sulfate,  $\gamma$ -phenylpropylguanidine sulfate,  $\delta$ -phenylbutylguanidine sulfate, *ar*-tetrahydro- $\beta$ -naphthylguanidine and (*dl*) *ac*-tetrahydro- $\beta$ -naphthylguanidine hydrochloride. The last two compounds gave a brown precipitate in the second series of tests.

*p*-Phenylenediguanidine dihydrochloride, 4,4'-diguanidodiphenyl dihydrochloride, and 4,4'-diguanidodiphenylmethane sulfate gave a brownish red color which could be distinguished readily from the typical red produced by guanidine.

*p*-Aminophenylguanidine, as its sulfate, nitrate, and hydroiodide, at every step in the reaction gave a red coloration which was so similar to that produced by guanidine that it was almost impossible to distinguish between them.

Four substituted guanidine derivatives,  $\alpha$ -naphthylguanidine hydrochloride, *ar*-tetrahydro- $\alpha$ -naphthylguanidine hydrochloride, *o*-tolylguanidine hydrochloride, and *p*-tolylbiguanide hydrochloride, in the final step of the reaction following addition of the nitric acid, gave a red color which was indistinguishable from that obtained with guanidine itself.

In view of these observations made on thirty-six different guanidine derivatives, not including various salts of the same base, it must be concluded that the Sullivan colorimetric test for the detection of free guanidine is not as highly specific as was first thought, since the reaction is positive with certain substituted guanidines. However, in consideration of the fact that the inter-

fering guanidine structures mentioned above normally would not be expected to be found in biological fluids, the specificity and value of the Sullivan test in clinical investigations still remain.

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## THE SPONTANEOUS DECOMPOSITION OF CYSTINE DIMETHYL ESTER

By ROBERT D. COGHILL

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(Received for publication, April 7, 1936)

In the course of some synthetic work which was attempted a number of years ago, the author had occasion to prepare a quantity of cystine dimethyl ester. Due to an interruption, the work had to be temporarily discontinued. When a few weeks later the problem was resumed, it was found that a profound decomposition had taken place in the ester. Whereas when originally prepared it had been a straw-yellow, viscous, practically odorless oil, it had now been changed into a dark brown semiliquid mass containing a profusion of sulfur and other crystals. Some of the sulfur crystals were as much as 2 cm. in length. In addition there was apparent a very strong mercaptan-like odor.

Since that time the experiment has been repeated many times. The reaction is largely completed in 6 weeks at laboratory temperatures, although some of the fractions which have been isolated continue to decompose for as long as a year. The decomposition may be hastened by carrying it out in boiling methyl alcohol, although it does not follow that the reaction proceeds along the same course in both cases.

It soon became evident that the decomposition was not a simple one, as was first suspected, but that several reactions were taking place simultaneously. The products which have been isolated up to this time are (A) sulfur, representing one-third of that originally present, (B) ammonium sulfate, (C) *dl*-alanine anhydride, (D) an ether-, alcohol-, and water-insoluble solid product, (E) an alcohol-soluble, ether-insoluble solid product, (F) a high boiling, sulfur- and nitrogen-containing oil.

## 420    Decomposition of Cystine Dimethyl Ester

### EXPERIMENTAL

*Preparation of Cystine Dimethyl Ester*—46 gm. of cystine dimethyl ester dihydrochloride were suspended in a liter of cold absolute methanol, and two molecular proportions of sodium methylate in methanol were added with continuous shaking, the mixture being cooled in an ice bath. The sodium chloride which precipitated was filtered off from the straw-colored solution and the methanol removed *in vacuo*. The last traces of salt were then removed by precipitation with ether, followed by filtration and removal of the solvent. The ether which was removed gave an alkaline reaction to wet litmus paper, and the residue, already containing small crystals of sulfur, smelled of ammonia, evidence of decomposition in this short time.

*Decomposition of Cystine Dimethyl Ester*—The ester so prepared was allowed to stand for 3 months at laboratory temperature. During this time it slowly darkened in color and became very viscous, almost solid. Large crystals of sulfur were apparent, and the whole smelled strongly of mercaptans. During the course of the decomposition the container was connected through glass tubing to a flask of dilute sulfuric acid. Analysis of this solution at the close of the experiment indicated that no nitrogen had escaped.

Partially to separate the products of decomposition, 200 cc. of methanol were added and the precipitate filtered off and washed with more methanol. The dried precipitate was extracted with freshly distilled carbon disulfide. Evaporation of the solvent left 2.93 gm. of sulfur, this representing 34.0 per cent of the sulfur of the original cystine.

The carbon disulfide-insoluble portion was divided into a water-insoluble portion (D) (0.6 gm.) and a water-soluble portion (B) (1.5 gm.). This latter fraction gave qualitative tests for the ammonium and sulfate ions and contained 20.4 per cent of nitrogen. The theoretical nitrogen content of ammonium sulfate is 21.2 per cent. The ammonium sulfate accounts for an additional 4.3 per cent of the sulfur and 8.5 per cent of the nitrogen of the cystine. The water-insoluble portion (D) remains unidentified. It dissolves in dilute sodium hydroxide to give a yellow-green fluorescent solution which decomposes upon standing to give sulfide ion, or if acidified at once, regenerates the original material. It is not cystine.

The alcohol was removed from the original methanol filtrate from the sulfur and ammonium sulfate, and the viscous brown residue was divided into ether-soluble and insoluble (E) fractions.

The ether-insoluble portion (E) (21 gm.) represents the chief decomposition product of the cystine. It is a yellow powder which slowly decomposes to give sulfur and a bad smelling oil. Its composition is not constant in different experiments, the sulfur varying from 16 to 25 per cent and the nitrogen from 11.8 to 14.5 per cent. This represents a N:S ratio of about 2:3. No means was found for the effective purification of this material. It yields no cystine either before or after hydrolysis with HCl.

The ether-soluble oil (3.5 gm.) was distilled with noticeable decomposition at 155° at a pressure of 17 mm., the distillate consisting primarily of a yellow oil. Accompanying this, however, was a quantity of white crystals which condensed in the side arm of the flask and the upper portion of the condenser, still more separating from the oil upon standing. These were filtered off and recrystallized from water. The analysis indicated that the material was *dl*-alanine anhydride as it melted with sublimation at 282° (corrected), which agrees with the known value.

*Analysis of dl-Alanine Anhydride*

	C	H	N
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Found .....	50.4	7.0	20.5
Theory.....	50.7	7.1	19.7

The oil, in order to free it of the *dl*-alanine anhydride and any basic materials, was dissolved in ether, repeatedly extracted with 2 per cent sulfuric acid, washed with water, dried, and redistilled. B.p. 155° at 17 mm. The final product gave the following analysis (two different preparations analyzed by Schoeller).

*Analysis of Oil from Decomposition of Cystine Ester*

	<i>per cent</i>	<i>per cent</i>
Carbon .....	42.9	41.3
Hydrogen.....	6.03	5.95
Nitrogen.....	3.37	3.57
Sulfur.....	25.3	23.6

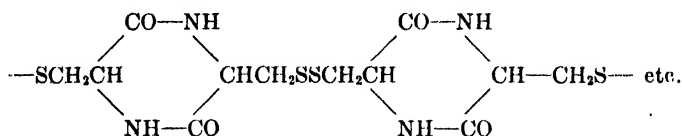


## 422 Decomposition of Cystine Dimethyl Ester

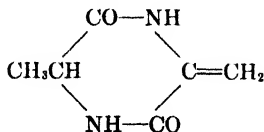
It is apparent that the preparations were not pure. Basing calculations on the average of these figures, one can derive the formula  $C_{14}H_{22}O_6NS_3$ . It might be questioned whether or not the nitrogen was an impurity. Analyses on other preparations not given here have shown essentially the same figures, and due to the washings with sulfuric acid, alanine ester or anhydride or any other basic nitrogenous substance would have been removed. The high viscosity and boiling point of the substance indicate a high molecular weight.

### DISCUSSION

Judging from the nature and multiplicity of the products formed during this decomposition, it must proceed in a variety of ways. It is suggested that the first step may consist in the formation of cystine anhydride. This would agree with the ease of formation of serine anhydride from its ester and the ease of anhydride formation in the case of the ester of dialanyl-*l*-cystine. Cystine anhydride should be a long chain molecule, a segment of which could be represented as follows:



It has been shown by Bergmann and Stather (1) that dialanyl-*l*-cystine dianhydride decomposes very quickly in the presence of 0.1 *N* sodium hydroxide in such a way that acidification produces sulfur, hydrogen sulfide, and 3-methylene-6-methyl-2,5-diketopiperazine (or its polymeric form)

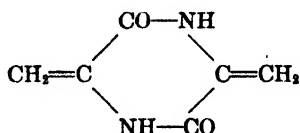


a compound which they had previously obtained in essentially the same manner from alanylserine anhydride. Dileucyl-*l*-cystine dianhydride was later shown to behave in the same way (2).

As Bergmann and Stather (1) have pointed out, the introduction

of cystine into a diketopiperazine ring makes the sulfur more labile than it is in the open chain peptide form, which in turn is more labile than free cystine itself. It is not surprising then that the introduction of two cystine units into one anhydride ring should predispose the molecule to the unusual instability which is apparent in the present case.

If we should assume that the molecule of cystine anhydride would decompose in the same fashion as the mixed anhydrides of cystine, we would expect to get sulfur, hydrogen sulfide, and 3,6-dimethylene-2,5-diketopiperazine



This would be expected to polymerize even more easily than the monomethylene compound of Bergmann and Stather, and it is possible that the substance (E) is derived by polymerization and reaction with sulfur from this dimethylene compound. Brand and Sandberg (3), in the course of preparing dialanyl-*L*-cystine dianhydride, obtained from their mother liquors a substance which had many of the characteristics of substance (E).

That the dimethylene compound is an intermediate product in the decomposition of cystine ester is amply supported by the isolation of *DL*-alanine anhydride, the racemic form being the one which would be expected from the reduction of the ethylenic linkage. The actual compound acting as the reducing agent is not known, but one would judge that there is a complicated system of oxidations and reductions going on, as evidenced by the formation of ammonium sulfate, the sulfur of which could only have been derived from cystine.

The nature of the oil boiling at 155° at 17 mm. is entirely unknown. It has a characteristic bad odor reminding one of burning protein. It is unstable to alkali, decomposing with the formation of sodium sulfide. Fischer and Dörpinghaus (4) mention the presence of a sulfur-containing oil in the residue from their distillation of the amino acid esters obtained from horn. This was easily decomposed by heat. Other isolated references in protein litera-

## 424 Decomposition of Cystine Dimethyl Ester

ture may be found indicating that cystine ester was behaving in this way when heated.

It is a well known fact that some proteins upon acid hydrolysis yield a small amount of free sulfur. As cystine, due to its dibasic nature, may occur in the protein molecule in the anhydride as well as in the peptide form, it is suggested that the heating of these peptide or anhydride linkages before complete hydrolysis has taken place may be the origin of the sulfur.

### SUMMARY

Cystine dimethyl ester slowly decomposes at laboratory temperature with the formation of sulfur, ammonium sulfate, *dl*-alanine anhydride, and two other products of complicated and unknown nature.

It is suggested that the course of this decomposition is through cystine anhydride and a hypothetical dimethylene diketopiperazine.

A reaction similar to this may be the origin of the small amount of free sulfur formed upon the acid hydrolysis of some proteins.

Acknowledgment is made of the help of Miss Marion Horn in a part of this work.

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## EVIDENCE CONCERNING TWO TYPES OF PLANT DIASTASE\*

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(Received for publication, December 13, 1935)

In making some studies of diastase, a sound mature wheat (Marquis) was germinated for 96 hours, dried, and the wheat before and after germination carefully separated into its three principal parts: bran, floury endosperm, and germ. Each finely ground material was digested for 1 hour in 5 cc. of water at 20° and without filtration was united with 50 cc. of suitable starch paste and allowed to digest at selected temperatures for 1 hour. This provides for the presence of all diastase of the portion taken, together with other materials with which it is naturally associated. The action of the diastase was stopped by solutions of sulfuric acid and sodium tungstate. The maltose formed was determined by the use of potassium ferricyanide much as described by Blish and Sandstedt.<sup>1</sup> This general procedure has been followed in many determinations some of which are described in this paper. The constancy and consistency of the results justify confidence in the methods.

Throughout the data presented in Figs. 1 to 3 and Tables I and II, two types of sugar-forming diastase are clearly indicated. One of those which predominates in the flour of sound mature wheat is favorably affected by a starch paste having a pH near 4.5 and less favorably affected by a starch paste having a pH near 6.2. The other shows the reverse condition. The latter type is the predominating diastase of the bran of many germinating cereals and of other parts of plants where active vegetation is taking place. The effects of change in pH on both diastases are pronounced but

\* Given in substance before the Biological Division at the meeting of the American Chemical Society at New York, April 22, 1935.

<sup>1</sup> Blish, M. J., and Sandstedt, R. M., *Cereal Chem.*, 10, 189 (1933).

different, especially at the higher temperatures of diastatic activity. In general, conditions at 60° are such that the ratio of the maltose produced by the flour diastase in the more acid of the above media to that produced in the less acid media is greater than 1. With the bran type of diastase it is less than 1.

Similar differences in pH effect are apparent in the diastase of many different materials (see Table I). The ratio as given, multi-

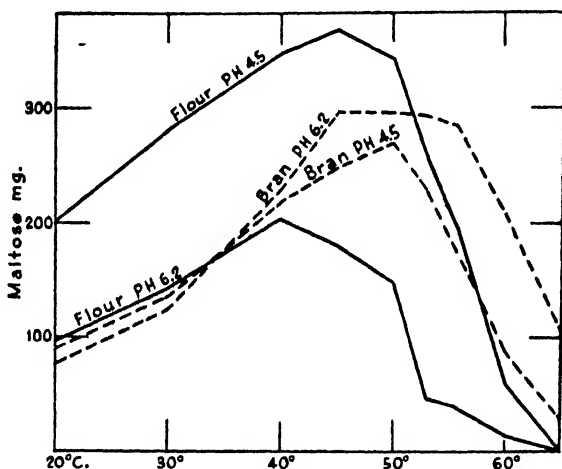


FIG. 1. Maltose formed at different temperatures in two types of starch paste by two types of diastases. The diastases used were those obtained respectively from 25 mg. of the bran of a wheat germinated for 96 hours, and from flour of the same wheat not germinated. The procedure followed is as given in the first paragraph. The media used were 2 per cent plain Lintner starch paste (pH near 6.2) and the same made to contain 0.05 mole each of acetic acid and anhydrous sodium acetate (pH near 4.5). 50 cc. of each paste were brought to the desired temperature, mixed with the diastase, and digested for 60 minutes.

plied by 100, for results at 60° is used as a convenient means of indicating which type of diastase predominates in the material described. That this may have analytical value when properly used was found when the sweet potato was studied and the two types of diastase located.

This method of differentiating the diastases, when applied to representative materials selected from different botanical families

TABLE I

*Diastatic Activity of Parts of Various Plant Products in Plain and Buffered Starch Pastes (pH Near 6.2 and 4.5)*

Kind of material	Amount	Maltose found				100 × ratio (b) : (a)
		20°		60°		
		Plain	Buf- fered	Plain (a)	Buf- fered (b)	
	mg.	mg.	mg.	mg.	mg.	
Mature grain						
Wheat (Turkey) germ . . . . .	50	28	28	83	23	28
Bran . . . . .	50	106	122	121	68	56
Endosperm* . . . . .	50	185	280	71	148	208
Indian corn, each of 5 parts† . . . .	25	0	0	0	0	0
Germinated grains						
Wheat, bran . . . . .	25	77	88	209	88	42
“ endosperm . . . . .	25	166	227	147	170	115
Barley, bran with husk (moist) . .	80	218	250	300	202	67
“ endosperm (moist) . . . . .	50	262	310	99	202	204
Naked barley, bran . . . . .	25	160	172	460	286	62
“ “ endosperm . . . . .	25	256	286	400	468	117
Indian corn, whole grain . . . . .	25	31	33	225	107	48
Immature and growing materials						
Wheat in late dough stage, bran .	20	150	93	160	51	32
Same, endosperm . . . . .	35	270	294	60	150	250
Emmer, bran . . . . .	45	156	182	159	156	98
“ endosperm . . . . .	20	258	405	119	247	207
“ chaff . . . . .	35	37	42	76	60	79
Oat heads in blossom . . . . .	25	60	54	80	48	60
Barley leaf . . . . .	40	4	8	42	16	38
Soy bean, not dried, cotyledons . .	12	50	49	242	290	120
“ “ bean skin . . . . .	50	6	6	40	33	82
“ “ leaf . . . . .	50	27	26	135	118	87
Arrowhead leaves . . . . .	400			342	88	26
Sweet potato, inside, dry . . . . .	6			117	367	314
“ “ sprouts, dry . . . . .	20			187	94	50
“ “ leaf, “ . . . . .	400			207	39	19
Partially purified diastase						
From bran of germinated wheat . .		81	96	172	82	48
“ flour of ungerminated wheat .		442	460	181	367	203
Taka-diastase, a fungus diastase . .	20	90	81	138	37	27

\* Endosperm refers in all cases to floury endosperm.

† Outer bran, inner bran, germ, layer beneath bran, remainder.

and supported by other determinations, shows what appears to be a continuous condition throughout a large part of the vegetable kingdom. In parts of some plants both types are found. In other plants only one. The presence of the two types throughout the normal existence of some grains, *e.g.* wheat, is clearly seen.

In Fig. 2 different starch media have been used in such a manner as to show the combined effects of varying temperatures and varying pH. The results in this diagram are in accord with what has been determined in various instances with other materials.

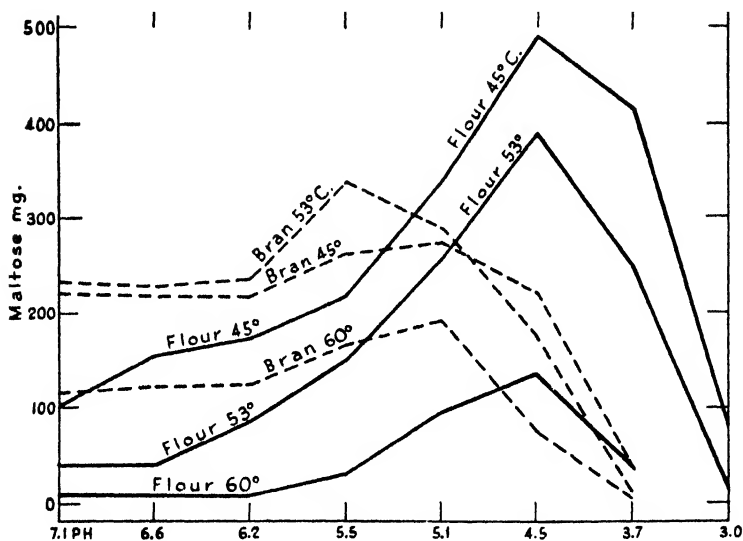


FIG. 2. The same two wheat diastase preparations used for Fig. 1. Starch pastes were made with Merck's Reagent starch. The pH values of the pastes were fixed by acetate buffers.

In Fig. 3 the effects of these conditions are shown on the two types of diastase found in parts of the sweet potato. The more favorable effect of the more nearly alkaline media on the diastase in the sprouts, as in wheat bran, and the reverse for the inside of the sweet potato, as in flour, are clearly seen.

Similar conditions are seen in Table II in which the results of these media with the two types of diastase from wheat partially purified are shown. Here characteristic differences throughout are apparent.

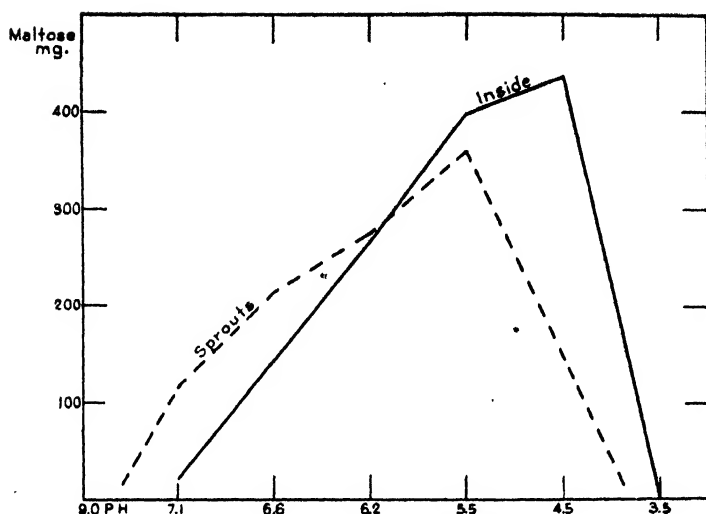


FIG. 3. Germinating sweet potato, inside and newly formed sprouts (without leaves or roots). The water extracts of the undried substance were in starch media as for Fig. 2 and at 60°. The solutions (5 cc.) were equivalent to 6 mg. of dry inside and 12 mg. of dry sprouts. See also Table 1.

TABLE II

*Diastatic Activity of Representative Alcohol Precipitates from Water Extracts of Bran from Germinated Wheat and of Flour from Ungerminated Wheat, As Modified by pH and Temperature*

The results are measured in mg. of maltose.

pH	45°		60°		65°	70°	
	Bran	Flour	Bran	Flour	Flour	Bran	Flour
9.0	0	0	1	0	2	0	0
7.1	94	328	43	12	14	12	0
6.6	139	466	125	78	21	64	6
5.5	207	559	220	323	87	137*	20
4.5	202	565	204	530	324	72	62
3.0	40	352	0	37	0	0	0

\* Examination of 125 mg. of meal of germinated Kaffir-corn and buck-wheat for diastase gave at this pH and 75° 69 mg. of maltose for the former and 37 mg. for the latter.



Upon further investigation of the two diastases disclosed by the above procedure it was found that at all temperatures the optimum pH for the flour type of diastase is near 4.5 and for the bran type near 5.3. At these more favorable pH values the maximum temperature for the flour type is near 70° and for the bran type near 75°. At other pH values the maximum temperature is lower for each. In the flour type of diastase the optimum temperature is near 45° and for the bran type near 53°.

By careful research not detailed here it was found that the newly formed maltose produced by each of these diastases has an upward mutarotation.

#### SUMMARY

Germinating barley, rye, and wheat and certain other plant products are shown to contain two types of sugar-forming diastase. These have different activities in starch media of different pH and at different temperatures. Where both diastases are present the amount of maltose produced at any one point as located by pH and temperature is a resultant of the combined action of the two. The divergence between the results of the two diastases acting in media of different pH is much greater at the higher temperatures of diastatic activities than at the lower. At 60° the difference is such that when the maltose produced by the one diastase in an acid starch paste of pH near 4.5 is divided by the maltose produced in equal time in less acid starch paste, pH near 6.2, the ratio will be materially greater than 1. This is the type characteristic of normal wheat flour and some other but not all reserve substances. It is here called *reserve* diastase. With the other diastase acting under like conditions, the ratio will be materially less than 1. This is the type developed during germination. It predominates in the bran of germinating cereals and in general in those plant parts most closely associated with active vegetation. It is here called *vegetative* diastase. A method of comparison based upon properties indicated may be used to trace the types of diastase in different plant substances. By it such widely different products as the wheat grain and the sweet potato are found to contain in selected parts each of the two types. The two types of diastase in the cereals named are present not only in the germinating grains but also in the immature seeds even in the earliest stages of their formation.

## THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

### XLV. ISOLATION OF $\alpha$ - AND $\beta$ -LEPROSOL\*

By J. A. CROWDER,† F. H. STODOLA,‡ AND R. J. ANDERSON

(From the Department of Chemistry, Yale University, New Haven)

(Received for publication, April 10, 1936)

An analysis of the acetone-soluble fat obtained from *Bacillus lepræ* is under way in this laboratory and, although the main investigation is not finished, we wish to report at this time concerning two interesting compounds isolated from the unsaponifiable matter. These compounds which possess phenolic properties will be designated by the names  $\alpha$ - and  $\beta$ -leprosols.

Both substances, so far as can be determined by analysis, are identical in composition and correspond to the formula  $C_{26}H_{46}O_2$  or possibly  $C_{26}H_{44}O_2$ . The leprosols are very similar in their reactions and yield similar derivatives. They only differ in solubility and in melting points.  $\alpha$ -Leprosols, the less soluble of the two compounds, melts at 100–101°, while  $\beta$ -leprosols melts at 84–85°.

The leprosols contain 1 oxygen atom in the form of a hydroxyl group, while the other oxygen atom is present as methoxyl; both compounds are easily brominated, forming monobromo derivatives, and both give monoacetates. The acetates are completely indifferent to bromine. The leprosols do not react with diazomethane but are readily methylated in alkaline alcoholic solution with dimethyl sulfate. In the Zeisel reaction both substances are demethylated, yielding crystalline dihydroxy compounds. The dihydroxy compounds form diacetates but do not react with

\* The present report is a part of a cooperative investigation on tuberculosis; it has been supported partly by funds provided by the Research Committee of the National Tuberculosis Association.

† Holder of a National Tuberculosis Association Fellowship at Yale University, 1933–35.

‡ Holder of a National Tuberculosis Association Fellowship at Yale University, 1934–36.

diazomethane nor can the original leprosols be regenerated by treatment with dimethyl sulfate.

The leprosols give no sterol color reactions but in alcoholic solutions they form slightly soluble addition compounds with digitonin.

The leprosols exhibit weakly acidic properties. From alcoholic solutions they are easily precipitated by water but alkaline alcoholic solutions remain clear on dilution with water and are precipitated only on acidification; however, the leprosols are removed completely from alkaline solutions by extraction with ether. Phenolic properties are indicated by color reactions: in aqueous alcoholic solutions blue-green colorations are obtained with ferric chloride and in the presence of a trace of ammonia deep blue colors are obtained with phosphomolybdic acid. The dihydroxy leprosols give similar color reactions.

The composition and the reactions of the leprosols would indicate that they contain a benzene nucleus and that the hydroxyl and the methoxyl group are in the ring. There may be one or more side chains but the nature or position of the side chains is unknown.

#### EXPERIMENTAL

##### *Isolation of Leprosols*

About 252 gm. of the neutral esters composing the acetone-soluble fat isolated from the leprosy bacillus, as described by Uyei and Anderson,<sup>1</sup> were saponified and yielded 55.5 gm. of unsaponifiable matter. The latter was a nearly black viscous mass which after standing for some time at room temperature deposited a small amount of solid substance. The solid material was only slightly soluble in petroleum ether, hence the total mixture was stirred up with 200 cc. of petroleum ether in which the thick oily portion dissolved. The insoluble solid matter was filtered off and washed with petroleum ether. The filtrate on cooling in ice water deposited a further small quantity of solid substance which was filtered off and combined with the main lot. The solid matter thus obtained was a brick-red powder which weighed 11.2 gm.

*Purification of Leprosols*—The crude product was dissolved in

<sup>1</sup> Uyei, N., and Anderson, R. J., *J. Biol. Chem.*, **94**, 653 (1931-32).

absolute methyl alcohol and the solution was decolorized with norit. By concentrating the solution and cooling, 8.6 gm. of a white amorphous powder were obtained. The substance was readily soluble in the ordinary organic solvents with the exception of petroleum ether but showed no tendency to crystallize, separating in globular particles as the solutions were cooled. After one precipitation the substance melted at 80° but several further precipitations raised the melting point to 91–93°. The properties indicated that we were dealing with a mixture which was not readily separated. The following properties were noted. A chloroform solution of the substance was optically inactive. In chloroform solution bromine was decolorized immediately and on evaporation of the solution a solid crystalline residue remained. The substance dissolved in concentrated sulfuric acid without apparent decomposition and on dilution with water a white precipitate separated.

*Separation of Leprosols by Means of the Acetyl Derivative*

The crude leprosol preparation was acetylated in pyridine solution with acetic anhydride and the acetate was isolated in the usual manner, yielding 8.6 gm. of a white product. Alcohol proved to be the best solvent for purification and after repeated recrystallization 1.9 gm. of substance with the constant melting point of 67–68° were obtained. A further quantity of 0.8 gm., having the same melting point, was recovered from the mother liquors. This top fraction will be designated  $\alpha$ -leprosol.

From the more soluble material contained in the mother liquors it was possible to isolate a second fraction which after repeated crystallization from alcohol had the constant melting point of 47–48° and which weighed 4.1 gm. This fraction will be designated  $\beta$ -leprosol. A further small quantity of  $\beta$ -leprosol was obtained from the last mother liquors, which after saponification was purified by means of the 3,5-dinitrobenzoate, as will be described later.

*$\alpha$ -Leprosyl Acetate*—The substance melted at 67–68°, solidified to a crystalline mass at 56°, and remelted at 69–70°.  $\alpha$ -Leprosol acetate was readily soluble in the ordinary organic solvents on warming, but on cooling these solutions only globular particles were obtained as a rule. It separated from alcohol in particles

having irregular edges, approaching a crystalline appearance. In chloroform solution the acetate did not absorb any bromine. For analysis the substance was dried *in vacuo* over dehydrite at 60° but there was no loss in weight.

*Analysis*—11.33 mg. substance: 11.40 mg. H<sub>2</sub>O and 32.40 mg. CO<sub>2</sub>

C<sub>26</sub>H<sub>44</sub>O<sub>2</sub>·COCH<sub>3</sub>(432). Calculated. C 77.78, H 11.11

Found. " 77.99, " 11.26

*Saponification*—0.5039 gm., 0.3946 gm. substance required 11.71 cc., 9.11 cc. 0.1 N KOH

C<sub>26</sub>H<sub>44</sub>O<sub>2</sub>·COCH<sub>3</sub>(432). Calculated. CH<sub>3</sub>CO 9.95, mol. wt. 432

Found. " 9.99, 9.93, " " 430, 433

*α-Leprosol*—The free *α*-leprosol was isolated from the saponification mixtures mentioned in the preceding paragraph and was twice precipitated from alcohol to which a little water was added. The substance separated in fine globular particles and the snow-white powder weighed 0.7 gm. The substance melted at 100–101°, solidified at 88°, and remelted at 100–101°.

*Analysis*—C<sub>26</sub>H<sub>44</sub>O<sub>2</sub>(390). Calculated. C 80.00, H 11.79

Found. " 80.03, 80.10, " 11.83, 11.90

*Bromo-α-Leprosol*—To 0.25 gm. of *α*-leprosol in 10 cc. of chloroform was added a chloroform solution of bromine in slight excess. The solution was washed with dilute sodium thiosulfate and with water, dried over sodium sulfate, filtered, and the solvent distilled off. The residue was recrystallized from methyl alcohol, separating in aggregates of fine colorless needles. The crystals weighed 0.3 gm. and melted at 66–67°, solidified in masses of needles at 59°, and remelted at 73–74°.

*Analysis*—C<sub>26</sub>H<sub>44</sub>O<sub>2</sub>Br(468.92)

Calculated. C 66.53, H 9.59, Br 17.04

Found. " 66.14, 66.26, " 9.67, 9.73, " 16.76, 16.60

The results of the analysis of *α*-leprosol and its derivatives indicate that the substance contains one hydroxyl group and that 1 hydrogen atom is easily replaced by bromine when the hydroxyl group is free. In order to determine the nature of the second oxygen atom attempts were made to prepare an oxime and a semicarbazone, but unchanged *α*-leprosol was recovered in both

cases. It was found, however, that one methoxyl group was present as determined by the Zeisel method.

*Analysis*— $C_{21}H_{41}\cdot OH\cdot OCH_3(390)$ . Calculated.  $OCH_3$ , 7.94  
Found. " 7.88, 7.92

*Demethylated  $\alpha$ -Leprosol*—The reaction mixture from the Zeisel determination was diluted with water, distilled with steam to remove phenol, and extracted with ether. The ethereal extract was washed with a dilute solution of sodium bicarbonate and with water, after which it was dried over sodium sulfate, filtered, and the ether was distilled off. The colorless residue was recrystallized from petroleum ether and was obtained as fine white silky needles. The substance melted at 113–114°.

*$\alpha$ -Leprosol Dimethyl Ether*—To 50 mg. of  $\alpha$ -leprosol, dissolved in 5 cc. of alcohol, were added 2 cc. of 50 per cent potassium hydroxide, 5 cc. of water, and 1 gm. of dimethyl sulfate. The solution was shaken for some time and then heated to boiling, cooled, diluted with water, and extracted with ether. The ethereal solution was washed with water, dried, filtered, and evaporated to dryness. The residue was recrystallized from methyl alcohol until the melting point was constant. The substance which crystallized in feathery forms consisting of fine needles melted at 78–79°, solidified at 73°, and remelted at 80–81°.

*Analysis*— $C_{25}H_{43}\cdot OCH_3\cdot OCH_3(404)$ . Calculated,  $OCH_3$ , 15.3  
Found. " 14.8

$\alpha$ -Leprosol could not be methylated with diazomethane. Attempts to prepare a nitro derivative did not lead to any well defined product.  $\alpha$ -Leprosol dissolved in glacial acetic acid and treated with nitric acid was apparently nitrated, but the substance was a soft yellow-colored solid which could not be obtained in crystalline form. The product was readily soluble in ether, acetone, and petroleum ether but less soluble in alcohol or methyl alcohol.

*$\beta$ -Leprosyl Acetate*—The product, obtained as already described, was readily soluble in the ordinary organic solvents on warming but did not separate in well defined crystals, globular particles being usually obtained. Ethyl alcohol proved the best solvent for purification. The dried substance was a white powder which melted at 47–48°, solidified at 45°, and remelted at 51–52°.

In chloroform solution the substance did not absorb any bromine and it showed no optical rotation.

*Analysis*—10.34 mg. substance: 10.27 mg.  $H_2O$  and 29.47 mg.  $CO_2$

$C_{26}H_{46}O_2 \cdot COCH_3(432)$ . Calculated. C 77.78, H 11.11

Found. " 77.73, " 11.11

*Saponification*—0.4168 gm. substance required 9.86 cc. 0.1 N KOH

$C_{26}H_{46}O_2 \cdot COCH_3(432)$ . Calculated,  $CH_3CO$  9.95; found,  $CH_3CO$  10.17

Calculated mol. wt. 432; found, 422

*$\beta$ -Leprosol*—The alkaline solution, after saponification of the acetyl derivative, gave no precipitate on dilution with water, but acidification caused a voluminous amorphous precipitate; however, the free alcohol could be extracted completely from the alkaline solution with ether.  *$\beta$ -Leprosol* exhibited, therefore, weakly acidic or phenolic properties. After the substance had been recovered from the saponification mixture, it was found to be readily soluble in the ordinary organic solvents on warming, but on cooling only globular particles separated, and from warm petroleum ether, on cooling slowly and scratching, fine colorless needles separated. From methyl alcoholic solution a white granular powder was obtained which melted at  $82-83^\circ$ , solidified to a crystalline mass at  $80^\circ$ , and remelted at  $85^\circ$ . The crystals from petroleum ether melted at  $84-85^\circ$ , solidified at  $80^\circ$ , and remelted at  $84-85^\circ$ .

*Analysis*—11.26 mg., 11.15 mg. substance: 11.88 mg., 11.77 mg.  $H_2O$  and 33.00 mg., 32.64 mg.  $CO_2$

$C_{26}H_{46}O_2(390)$ . Calculated. C 80.00, H 11.79

Found. " 79.93, 79.84, " 11.81, 11.81

*Monobromo- $\beta$ -Leprosol*—Although the acetyl derivative was perfectly indifferent to bromine, the free  *$\beta$ -leprosol* absorbed 1 atom of bromine immediately with liberation of hydrobromic acid. The bromination was carried out in chloroform solution as described under  *$\alpha$ -leprosol*, 0.2 gm. yielding 0.25 gm. of the bromo derivative. The substance did not crystallize from solvents but separated in the form of globular colorless particles. For purification the substance was dissolved in hot methyl alcohol; on cooling, a white granular powder separated. The substance melted at  $42-43^\circ$ , solidified to a crystalline mass at about  $40^\circ$ , and remelted at  $45-46^\circ$ .

*Analysis*—10.425 mg. substance: 8.75 mg.  $H_2O$  and 25.05 mg.  $CO_2$   
20.47 mg. substance: 8.35 mg. AgBr

$C_{10}H_{11}O_2Br$  (468.92). Calculated. C 66.53, H 9.59, Br 17.04  
Found. " 65.44, " 9.35, " 17.36

*$\beta$ -Leprosyl-3,5-Dinitrobenzoate*—The material contained in the mother liquors from which the  $\beta$ -leprosyl acetate had been crystallized was recovered and saponified with alcoholic potassium hydroxide. The crude product thus obtained was dissolved in pyridine and treated with a slight excess of 3,5-dinitrobenzoyl chloride dissolved in warm pyridine. The solution was warmed to  $50^\circ$  for 0.5 hour, after which it was allowed to stand at room temperature for 2 days, at the end of which time it was poured into dilute hydrochloric acid. The precipitate which formed was filtered off, washed with water, dissolved in ether, and the ethereal solution was washed with a solution of sodium carbonate and with water. On evaporation of the ether a yellow residue was obtained which crystallized from alcohol in very fine long yellow needles. After three recrystallizations the substance melted at  $86-87^\circ$  and the melting point was not changed on further recrystallization.

*Analysis*—11.06 mg. substance: 8.13 mg.  $H_2O$  and 27.46 mg.  $CO_2$   
 $C_{21}H_{15}O_2 \cdot COC_6H_3(NO_2)_2$  (584). Calculated. C 67.81, H 8.21  
Found. " 67.71, " 8.23

The phenylurethane of  $\beta$ -leprosol was prepared, but since it could not be obtained in crystalline form, it was saponified and the free alcohol was recovered.

Experiments were made to determine the nature of the second oxygen atom in  $\beta$ -leprosol. Attempts to prepare the oxime and semicarbazone were unsuccessful and the unchanged  $\beta$ -leprosol was recovered in both cases.

It was found, however, in the Zeisel reaction, that a volatile iodide was liberated, corresponding to one methoxyl group.

*Analysis*— $C_{25}H_{43}OH \cdot OCH_3$  (390). Calculated.  $OCH_3$  7.94  
Found. " 8.1, 8.4

The alkyl halide was characterized in the usual way, an alcohol solution of trimethylamine being used. 0.1035 gm. of  $\beta$ -leprosol yielded 46.3 mg. of white crystalline tetramethylammonium iodide.



*Analysis*—11.09 mg. substance: 6.08 mg.  $H_2O$  and 9.84 mg.  $CO_2$ ,  
 $(CH_3)_4NI(201)$ . Calculated. C 23.88, H 5.97  
 Found. " 24.20, " 6.14

*Dihydroxy- $\beta$ -Leprosol*—The demethylated  $\beta$ -leprosol could be isolated from the Zeisel reaction mixture. Since phenol was used to dilute the hydriodic acid, the reaction mixture was diluted with water and distilled with steam until the phenol was removed, after which the solution was extracted with ether. The ethereal extract was washed with water and with dilute sodium bicarbonate solution, treated with norit, filtered, and evaporated to dryness. The nearly colorless residue was twice recrystallized from large volumes of petroleum ether and was obtained as fine colorless needles, m.p. 104–105°. Recrystallization caused no change in the melting point.

*Analysis*—9.895 mg. substance: 10.25 mg.  $H_2O$  and 28.74 mg.  $CO_2$ ,  
 $C_{25}H_{44}O_2(376)$ . Calculated. C 79.78, H 11.70  
 Found. " 79.21, " 11.59

*Attempts to Methylate Dihydroxy- $\beta$ -Leprosol*—The demethylated  $\beta$ -leprosol did not react with diazomethane, the unchanged substance being recovered. On treatment with dimethyl sulfate in alkaline dilute alcoholic solution, a product was obtained which was extremely soluble in all of the usual organic solvents including petroleum ether and it was impossible to crystallize the substance. The results thus indicate that  $\beta$ -leprosol cannot be regenerated by methylation of the dihydroxy compound.

That the demethylated  $\beta$ -leprosol contained two hydroxyl groups was shown by the formation of a diacetyl derivative.

*Diacetyl Derivative of Dihydroxy- $\beta$ -Leprosol*—The demethylated  $\beta$ -leprosol, 50 mg., was acetylated in pyridine solution with acetic anhydride and the reaction product was isolated by extraction with ether. The substance was very soluble in the usual organic solvents and it did not crystallize. The product was dissolved in a little alcohol and the solution was cooled in a freezing mixture. A white amorphous powder was obtained which weighed 25 mg. and melted at 48°.

*Analysis*— $C_{25}H_{42}O_2(COCH_3)_2(460)$ . Calculated. C 75.65, H 10.43  
 Found. " 75.25, " 10.39

*Methylation of  $\beta$ -Leprosol*— $\beta$ -Leprosol did not react with diazomethane in ethereal solution but in alkaline dilute alcoholic solution methylation was quantitatively effected with dimethyl sulfate. The reaction product separated from the solution almost immediately as a white crystalline precipitate. The dimethyl ether was decidedly less soluble in alcohol than was  $\beta$ -leprosol itself but it only separated in the form of globular particles. It was very soluble in ether and in petroleum ether and from very concentrated solutions needle-shaped crystals separated. The substance was recrystallized from a mixture of ether and methyl alcohol, separating in bundles of delicate colorless needles. The crystals softened at  $64^{\circ}$  and melted at  $68$ – $69^{\circ}$ , solidified at  $67^{\circ}$ , and remelted at  $70^{\circ}$ . The globular particles separating from an alcoholic solution had the same melting points.

*Analysis*—10.50 mg. substance: 11.26 mg.  $H_2O$  and 30.74 mg.  $CO_2$

$C_{25}H_{41} \cdot OCH_3 \cdot OCH_3 (404)$ . Calculated. C 80.19, H 11.88

Found. " 79.84, " 12.00

*Molecular Weight*—1.245 mg. substance dissolved in 9.705 mg. camphor gave a depression of  $12.5^{\circ}$ . Found, mol. wt. 411

#### SUMMARY

Two new alcohols,  $\alpha$ - and  $\beta$ -leprosol, having phenolic properties, have been isolated from the unsaponifiable matter of the neutral fat from *Bacillus lepræ* and some of their properties have been determined.



# THE EFFECT OF INGESTED COTTONSEED MEAL UPON THE DISTRIBUTION OF THE CONSTITUENT FATTY ACIDS OF GOAT MILK

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The constituent fatty acids and their distribution in a sample of goat milk fat produced under a regulated dietary regimen have been reported in a previous investigation (1). In addition to decenoic, tetradecenoic, oleic, and arachidonic acids, as reported by Bosworth and coworkers (2, 3), in butter fat from cow's milk, approximately 2 per cent of palmitoleic (hexadecenoic) acid was found. The presence of linoleic acid could not be detected by the chemical methods available. This observation agrees with that of Bosworth and Brown. Hilditch and coworkers (4, 5) reported as much as 5 per cent linoleic acid, although no crystalline tetrabromide derivatives of this acid were obtained. However, Hilditch and Jones (6) reported traces of a bromide insoluble in ether, which darkened at 170–180° without definitely melting. This bromide was probably a mixture of isomers of octabromoarachidic acid. Dhingra (7) reported the same acids present in the milk fat of Indian goats as were reported by Hilditch and coworkers in butter fat from cow's milk, although in different proportions.

Despite the divergence of opinion and results as to the presence or absence of decenoic, tetradecenoic, palmitoleic, and arachidonic acids, whose presence collectively probably does not exceed 5 per cent, there is general agreement that the fat of goat milk contains greater amounts of capric, as well as caprylic and caproic acids, than does the fat of cow's milk. Certain objectionable qualities cited in a few instances have been attributed to the increased amounts of these acids of lower molecular weight.

The question naturally arises as to whether the composition of

goat milk fat can be so modified as to increase the content of acids of higher molecular weight at the expense of the acids of lower molecular weight. This would seem possible from the work of Eckles and Palmer (8) who showed that the presence of either cottonseed oil or meal in the diet of dairy cows lowered the Reichert-Meissl and saponification numbers and raised the iodine number and melting point of the butter fat. Ellis, Rothwell, and Pool (9) showed that cottonseed oil in the diet of hogs caused an increase in stearic and linoleic acids of the body fat at the expense of palmitic and oleic acids. In view of the probable replacement of a portion of the acids of low molecular weight, including butyric, caproic, caprylic, and capric, with acids of higher molecular weight in butter, as indicated by the findings of Eckles and Palmer, it was thought possible that the inclusion of cottonseed oil or meal in the diet would increase the amount of linoleic acid if that acid is a constituent of butter fat produced under normal conditions.

Accordingly a study of the effects produced by the inclusion of different levels of cottonseed meal in the diet of lactating goats was undertaken. Milk fat samples representing the different levels of meal feeding were compared as to their general chemical constants. Separations of the component fatty acids were then made on a sample from the basal diet feeding and on a sample from the diets containing cottonseed meal.

#### EXPERIMENTAL

*Feeding*—The experimental feeding was divided into four periods of 14 days each, in which the daily rations (Table I), per animal, were fed to a group of three Toggenberg and Saanen goat does.

The grain mixture consisted of 8 parts of wheat, 4 parts of oats, 2 parts of wheat bran, and 1 part of linseed oil meal. The animals were allowed pasture grazing throughout the feeding periods, which lasted from August 21, 1932, to November 18, 1932. The milk samples used for analysis were collected from the 11th to the 14th day of each period.

*Preliminary Examination of Samples*—Butter fat was obtained from the milk samples by churning the separated sweet cream, and filtering the melted butter. Some of the general analytical characteristics of samples from each period were determined, the

results of which were given in Table II. A lowering of the saponification number, Reichert-Meissl number, iodine number, water-soluble acids, and thiocyanogen number and an increase in Polenske value were observed for the periods of cottonseed meal feeding. Since the changes in values were greater in Periods II and III than in Period IV, even though the meal was fed at a higher level in the latter, it would seem that there was some recovery from the initial effects. This observation is in accordance

TABLE I  
*Experimental Daily Rations*

Period	Grain mixture	Cottonseed meal	Alfalfa hay
	<i>pounds</i>	<i>pounds</i>	<i>pounds</i>
I. Basal.....	1.50	0.00	3
II. Low cottonseed meal.....	1.00	0.50	3
III. Medium " ".....	0.75	0.75	3
IV. High " ".....	0.50	1.00	3

TABLE II  
*Fat Constants on Samples of Butter Fats*

Period No	Saponification No.	Soluble acids*	Reichert-Meissl No.	Polenske value	Iodine No.	Thiocyanogen No.
		<i>per cent</i>				
I	234.8	3.8	25.3	7.6	32.2	27.2
II	233.4	3.2	22.3	7.9	27.6	21.7
III	231.8	3.2	20.5	8.4	29.0	23.3
IV	231.9	3.4	22.3	7.8	31.1	24.7

\* Calculated as butyric acid.

with that of Eckles and Palmer (8). However, these authors reported an increase in iodine number due to the feeding of the meal.

*Separation and Estimation of Fatty Acids*—The method developed and used by Hilditch and coworkers (4, 6) to obtain a comparison of the acids of butter fat produced by the inclusion of coconut and soya cake in the diet, was used in the present investigation. The method involves (a) steam distillation and subsequent extraction and separation of the volatile acids by ordinary distillation,

TABLE III  
Summary of Total Weights and Percentages of Individual Acids Present in Samples from Periods I and III

Acid	Period I. No cottonseed meal					Period III With cottonseed meal				
	Weights of acids in primary fractions			Total acids		Weights of acids in primary fractions			Total acids	
	Volatile gm.	Solid gm.	Liquid gm.	Weight gm.	per cent	Volatile gm.	Solid gm.	Liquid gm.	Weight gm.	per cent
Butyric.....	12 37			12 37	2 4	11 72			11 72	2 1
Caproic.....	12 80			12 80	2 5	11 91			11 91	2 1
Caprylic.....	4 77		2 54	7 31	1 4	5 86		4 67	10 53	1 8
Capric.....	2 57	0 38	34 05	37 20	7 2	3 23	6 69	40 18	50 10	8 9
Lauric.....	1 86	6 98	11 71	20 55	3 9	2 75	13 47	15 55	31 77	5 6
Myristic.....		38 65	15 45	54 10	10 4		49 26	15 15	64 41	11 4
Palmitic.....		173 10	5 38	178 48	34 4		166 32	4 12	170 44	30 1
Stearic*		40 45		40 45	7 8		54 80		54 80	9 7
Decenoic.....	0 20		0 91	1 11	0 2	0 24	0 17	1 10	1 51	0 3
Tetradecenoic.....		0 11	1 75	1 86	0 4		0 64	1 68	2 32	0 4
Palmitoleic.....		3 89	9 92	13 81	2 7		3 90	11 86	15 76	2 8
Oleic.....		20 43	110 60	131 03	25 2		38 95	92 74	131 69	23 2
Arachidonic.....			7 84	7 84	1 5			9 12	9 12	1 6
Total.....	34 57	284 19	200 15	518 91	100 0	35 71	384 20	196 17	566 08	100 0

\* Calculated as stearic acid, although very small amounts of higher saturated acids were present.

(b) lead salt-alcohol separation of the non-steam-volatile acids into liquid and solid acids, and (c) fractional distillation at low pressure of the methyl esters of the solid and of the liquid acids. The composition of each distilled fraction is determined by calculations based on the saponification and iodine numbers.

From the distillation data and analysis of the fractions, the weights of the various acids present in the steam-volatile, solid and liquid fractions, and the percentages of the constituents of the total mixed acids were calculated. These data are given in Table III.

The same assumption in regard to the unsaturated acids as was made in the previous investigation (1) was employed in the calcu-

TABLE IV  
*Brominated Acids from Higher Boiling Fractions of Liquid Esters*

Period No	Weight for bromination	I No.	Petroleum ether-soluble bromides*		Ether-soluble bromides†		Ether-insoluble bromides‡	
			Weight	Bromine	Weight	Bromine	Weight	Bromine
	gm.		gm.	per cent	gm.	per cent	gm.	per cent
I	14 02	100 3	21.53	37.7	0.2190	65.3	0.2359	68.3
III	23 65	104 1	36 62	38 3	0.3713	65.8	0.4049	67.7

\* Oily dibromides; analyses indicate dibromostearic acid.

† These bromides appeared to soften and turn black at 150–155°.

‡ The bromides started to darken at about 180°; at 220° they appeared to soften, shrink to the center of the tube, and blacken at 220–225°.

lations reported here; that is, the unsaturated esters distil chiefly with the saturated esters of the same number and of 2 less C atoms.

*Bromination of Higher Unsaturated Fractions*—The acids obtained from the fractions of liquid esters whose iodine numbers were greater than that of oleic acid were brominated in petroleum ether. The solid bromides thus obtained were separated into ether-soluble and ether-insoluble fractions. Melting points and bromine analyses were made on these fractions, the results of which are given in Table IV.

#### DISCUSSION

The estimations of the amounts of each acid present, as given in Table III, showed that the inclusion of cottonseed meal in the diet



in Period III produced an increase in capric, lauric, myristic, and stearic acids at the expense chiefly of palmitic and oleic acids. The slight lowering in butyric and caproic acids was probably more significant from the standpoint of the lowering of the Reichert-Meissl number than from the actual calculated results. The Polenske values were in conformity with the increase in capric and lauric acids. From the results of the brominations, the presence of arachidonic acid was definitely shown. Some doubt remains concerning the identity of the ether-soluble bromides, although no linoleic tetrabromides were isolated. As in the previous publication (1), the bromine content was low for an isomer of octabromoarachidic acid (theory 67.8 per cent Br). This questionable bromide may be a mixture of bromides of  $C_{20}$ ,  $C_{22}$ , and  $C_{24}$  acids, or possibly a brominated  $C_{20}$  acid that had undergone slight change during the distillation and subsequent treatment.

Since no linoleic tetrabromides were isolated even from the most unsaturated fractions of the sample obtained from the cottonseed meal feeding, it is considered doubtful whether linoleic acid is a normal constituent of goat milk fat.

#### SUMMARY

Samples of goat milk fat were examined to determine the changes in fatty acid distribution produced by the inclusion of cottonseed meal in the ration.

The butter fat constants indicate a decrease in butyric and unsaturated acids and an increase in acids concerned in the Polenske values.

A study of the fatty acid distribution by the methyl ester distillation method substantiated the general information obtained from the butter fat constants and indicated the particular acids involved. Thus an increase in capric, lauric, myristic, and stearic acids at the expense chiefly of palmitic and oleic acids was observed as caused by the inclusion of cottonseed meal in the ration.

No evidence of the presence of linoleic acid was observed, although acids of the arachidonic type were found.

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# THE DETERMINATION OF TOTAL BASE IN BLOOD AND OTHER BIOLOGICAL FLUIDS BY THE ELECTRODIALYSIS METHOD OF ADAIR AND KEYS

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The method of Adair and Keys (1934) for the determination of base by electrodialysis would seem to be well suited for general use with biological fluids in which, frequently, the concentration of total base is important. In the original communication it was shown that errors of no more than 1 to 2 per cent are made with single determinations on samples containing a total of the order of 0.01 milli-equivalent of base and that the presence of as much as 10 per cent protein in the sample does not interfere with the ease and accuracy of the analysis. The bases tested were  $\text{NH}_4^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{++}$ , and mixtures of these ions. Only calcium showed an incomplete recovery, approximately 95 per cent, when pure  $\text{CaCl}_2$  solutions were used.

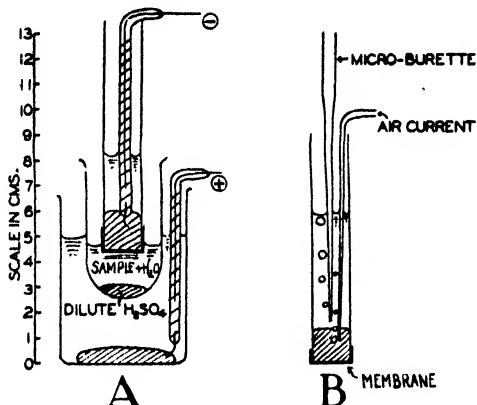
Some trials with normal horse serum gave satisfactory results, but no serious effort was made to check the absolute accuracy of the method with such fluids nor to develop standard procedures for routine laboratory use. The present paper describes certain modifications in the procedure and apparatus and presents results obtained in routine use with blood and plasma.

The essential feature of the method is that base ions are electro-dialyzed from the solution being analyzed across a collodion or cellophane membrane to negatively charged mercury above which stands a known amount of standard acid. When the dialysis is complete, the circuit is broken, the base-mercury amalgam decomposed by shaking with the acid, and the excess acid is titrated without removing it from the vessel.

*Apparatus and Reagents*

Figs. 1, *A* and 1, *B* show the apparatus arrangement; *A*, the assembly for electrodialysis, and *B*, the titration arrangement. It is wise to adhere to dimensions similar to those given in Fig. 1; otherwise the adjustment of fluid levels and volumes may be troublesome.

The membrane may be of collodion, prepared as described by Adair and Keys, but cellophane membranes are preferable because they can be allowed to dry, they are uniform, and, as we have found, very easy to prepare. The cathode vessel is a tube 1.5 cm. in diameter and 10 cm. long. Cellophane (du Pont No. 300 plain

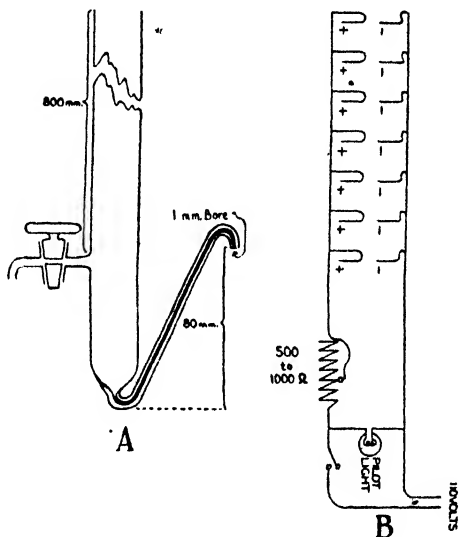


FIGS. 1, *A* AND 1, *B*. *A*, assembly for electrodialysis of base. *B*, arrangement for titration. Cross-hatching indicates mercury.

transparent) is cut into pieces about 5 cm. square and soaked in distilled water for 10 minutes or longer. A piece of this softened cellophane is then blotted, placed over the end of the tube, and molded in place briefly with some force. This stretching may be repeated once or twice in the next few minutes while the cellophane is drying. Finally, the edges are cut off, leaving a rim of several mm. which will adhere firmly to the glass when dry. As a final precaution, after the cellophane has dried thoroughly, a few drops of collodion solution (made up of equal parts of du Pont collodion, anhydrous ether, and ethyl alcohol) are run around the tube, spreading over the cellophane rim and the adjacent glass. (Flat pieces of incompletely dried collodion can easily be molded and pre-

pared in a similar way, *cf.* Keys and Taylor (1935).) In this way several dozen cathode vessels may be prepared with cellophane membranes in an hour.

The mercury used in both anode and cathode vessels must be pure. With a moderately good grade of mercury originally, it is cleaned sufficiently by spraying it twice through a long column of 5 per cent nitric acid and then through a number of changes of distilled water. It is most convenient for both acid and water wash-



FIGS. 2, A AND 2, B. A, mercury cleaning tube. B, wiring diagram for a seven unit system for electrodialysis.

ing to use tubes, like that illustrated in Fig. 2, A, which deliver the mercury without contamination by the washing fluid.

The distilled water used to dilute the solution to be analyzed must likewise be pure. In most laboratories the chief objection to the use of the stock distilled water is its frequent appreciable content of ammonia. This is easily corrected by redistillation in glass after addition of a few drops of phosphoric acid per liter.

Either HCl or  $\text{H}_2\text{SO}_4$ , 0.02 N, will serve for the standard acid. The concentration of the NaOH or KOH solution with which the titration of the excess acid is made should be about 0.2 N if the Rehberg microburette (0.1 cc. capacity) is used. Perfectly satis-

factory results are obtained, however, by the use of 0.01 N alkali in a 2 cc. burette divided in 200 parts. In the latter case the delivery tip of the burette should be drawn out to a fine capillary so that it may be safely used under the surface of the acid during the titra-

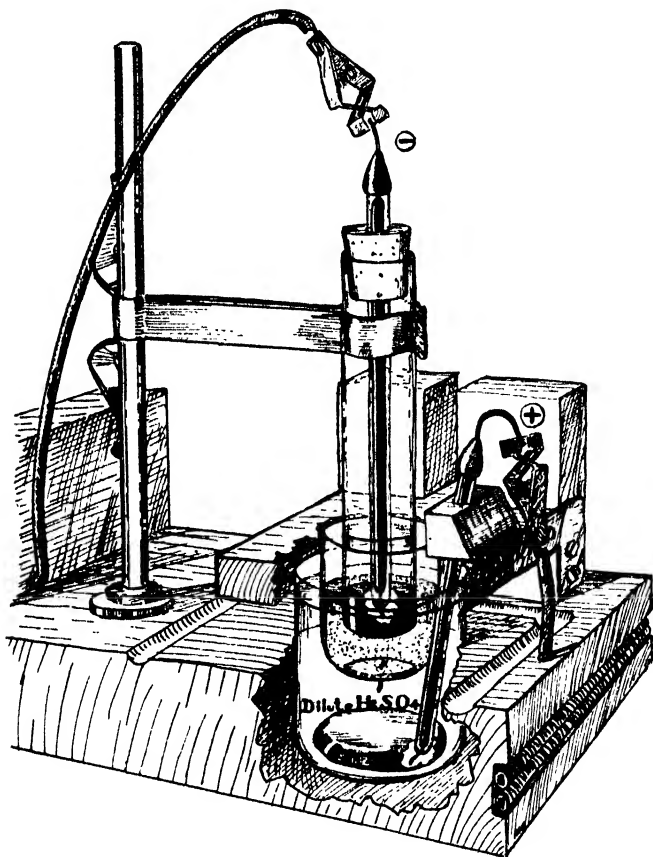


FIG. 3. Sketch of mounting of one unit in electroanalysis apparatus

tion. With either burette stirring is best done by a current of air (preferably washed through dilute  $\text{H}_2\text{SO}_4$ ) delivered by a capillary tube which dips under the surface of the mercury during the titration, as shown in Fig. 1, B.

Fig. 2, B gives a wiring arrangement for the simultaneous elec-

trodialysis of six samples and a blank. A unit of the assembly for six determinations is shown in Fig. 3.

We have used pipettes calibrated to contain 0.1 or 0.2 cc. for the measurement of the sample to be analyzed. After delivery these are rinsed with distilled water twice, the rinsings being added to the sample in the anode vessel. The measurement of the acid into the cathode vessel is most quickly and accurately done with a syringe-pipette of the Krogh-Keys type (1931), but an ordinary glass pipette to deliver will do..

### Procedure

In the anode vessel are placed 0.5 cc. of mercury and a standard amount—2 to 20 cc.—of distilled water to which is added the

TABLE I

*Summary of Amounts and Concentrations of Reagents to Be Used for Analysis of Total Base in Various Materials*

The alkali concentration was 0.2 N in the Rehberg burette; 0.01 N in the 2 cc. burette.

Material	Amount of material used	Amount of 0.02 N acid to use
		cc.
Whole blood, plasma, serum, cells, etc.	0.2 cc.	2.0
	0.1 "	1.0
Tissue	Ash from about 0.2 gm.	2.0
Urine	0.1 cc.	2.0
Blank		1.0

sample, generally 0.2 cc. of serum, plasma, whole blood, or cells. Smaller amounts, 0.1 cc. or less, can be used if these volumes are accurately measured; larger amounts may give low results, especially if more than 0.5 cc. is used. For urine we use 0.1 cc. or 1 cc. of a 10-fold dilution.

In the cathode vessel are placed 1.5 cc. of mercury and the standard acid (see Table I for the amount). The cathode vessel is then lowered into the fluid of the anode vessel, the electrode, rinsed with distilled water, is placed so that good contact is made with the mercury in the cathode, and the current, 110 volts D.C., is switched on.

In the outer vessel of the anode the rate of formation of bubbles



in the dilute  $\text{H}_2\text{SO}_4$  indicates the rate of dialysis. Electrolysis is begun with the rheostat on the lowest position; after a few minutes the rheostat may be opened wide or cut out of the circuit. The completion of electrolysis is signalized by the cessation of bubbling in the outer anode vessel. With No. 300 cellophane membranes, electrolysis is generally complete in about 25 minutes, but a standard period of 1 hour is safest.

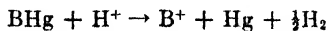
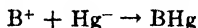
At the end of electrolysis the cathode vessel is lifted out of the anode fluid and the current switched off. The electrode is rinsed down with 1 cc. of distilled water as it is being removed from the cathode vessel.

The decomposition of the mercury-base amalgam is completed by gentle agitation by hand, by a slow moving mechanical shaker, or by a slow current of air from a capillary tube dipping into the mercury. 5 minutes are ample for this purpose. Titration is carried out as described by Rehberg (1925) if the 0.1 cc. micro-burette is used. If the 2 cc. burette is used, the tip should dip into the acid. Methyl red, 0.02 per cent in 60 per cent alcohol, is used as indicator, and 0.02 cc. is sufficient. The amount should be kept constant in all determinations; for most purposes a single drop from a small bore dropping pipette is constant enough. The best end-point is the final and permanent disappearance of all red color.

The cellophane on the anode vessel may be used repeatedly, but it gradually becomes brittle and coated with a relatively impermeable layer of insoluble by-products. We find it best to use a membrane only four or five times before replacing it. The cathode vessels with the cellophane membranes are washed out with water, soaked in distilled water, rinsed with glass-distilled water, and stored dry or wet as preferred. If collodion membranes are used, they must never be allowed to dry at any time, and are stored in distilled water in a refrigerator.

It is advisable to run one or more blank determinations with every series of analyses. These are made as indicated in Table I with the same amounts of mercury and water as are used with the samples.

The calculation is indicated by the reactions



In general, then

Concentration (milli-equivalent per liter) =

$$1000 \frac{[\text{acid}] \times (\text{cc. acid} - \text{cc. acid used by blank}) - [\text{alkali}] \text{ cc. to titrate}}{\text{cc. sample}}$$

where the brackets indicate normality.

### Results

The present method responds not only to fixed bases but also to  $\text{NH}_4^+$  and so is not perhaps strictly comparable to the methods of Fiske (1922), Stadie and Ross (1925), Van Slyke, *et al.* (1927), and others which have disregarded ammonium ions. Since in (fresh) blood and plasma the concentration of ammonium ions is only of the order of 0.1 to 0.2 milli-equivalents per liter, the difference between the present and the older methods would be small; in urine, however, the two methods cannot be compared directly. Because of the complexity of the older methods, and the fact that there is no reason to assume them to yield incontrovertibly "standard" results, it would appear to be best to evaluate the accuracy of the present method by (1) the agreement of duplicates, (2) the recovery of known base added to the solutions, and (3) the correspondence between the analytical results for total base and the sum of separately determined cations.

Table II illustrates the agreement between duplicates; the figures are taken at random from routine analyses in various experiments. In Table III are given a few representative cases where known salt solutions were added to plasma.

The base not accounted for by Na, K, Ca, and Mg (last column, Table IV) represents the sum of the errors in the several analyses plus the undetermined bases. It is difficult to evaluate these latter. Preformed ammonia is small but not entirely negligible (Bisgaard and Noevig, 1923; Klisiecki, 1923; Parnas and Heller, 1924; Benedict and Nash, 1926; Folin, 1932). The sum of the bases in Table V is of the order of 0.5 to 0.7 milli-equivalent per liter. This leaves an average of about 1 milli-equivalent of base representing either a systematic error or bases other than those listed in Tables IV and V. While there is no evidence on the

matter, it is possible that this represents ammonia derived from deamination of amino acids.

TABLE II

*Agreement between Duplicates with 0.2 Cc. Samples of Plasma, Whole Blood, and Cells*

The values are given in milli-equivalents of base per liter.

Material		Individual values	Mean
Dog	Plasma A*	178.2, 178.2	178.2
"	" B*	166.8, 166.9	166.85
"	" C*	170.9, 170.8	170.85
Ox	" †	164.2, 165.0, 164.5, 164.2	164.5
Human	" A†	150.5, 149.5, 150.6	150.2
"	" B†	156.4, 155.7, 155.0, 157.8, 156.2, 156.6	156.3
"	" C†	157.1, 157.0, 157.7, 157.5, 157.1, 158.0	157.4
"	whole blood†	131.5, 131.0, 131.8, 131.9, 132.5, 131.7	131.7
"	red cells†	112.0, 111.1, 112.7, 112.9	112.2
"	" " †	116.2, 117.0, 117.6	116.9
"	" " *	116.8, 116.2	116.6
Blank (H <sub>2</sub> O + Hg).....		2.0, 2.4, 2.4, 2.1, 2.5, 2.2	2.3

\* From work experiments.

† Oxalated.

‡ Heparinized blood from resting subjects.

TABLE III

*Mean Recovery of Known Base Added to Water and to Blood Plasma*

The values are given in milli-equivalents per liter.

Added base	Known	Found	Known	Found	Known	Found
H <sub>2</sub> O + NaCl.....	171.4	170.7	100.2	100.0	50.1	50.3
" + " + KCl + CaCl <sub>2</sub> ....	156.6	156.3	78.3	78.0		
Plasma + NaCl.....	40.0	39.5	40.0	40.0	171.4	173.0
" + " + KCl + CaCl <sub>2</sub> ....	39.2	39.0	39.2	39.6	19.6	19.2
H <sub>2</sub> O + K <sub>2</sub> SO <sub>4</sub> .....	46.3	46.6	92.6	93.0		
Cells + K <sub>2</sub> SO <sub>4</sub> .....	46.3	47.0	46.3	47.3		

A few general remarks about the method may be of use. If the volume of the titration mix at the end of the titration is larger than 5 cc., there may be difficulty in judging the end-point. The formation of ammonia in shed blood on standing seems to come chiefly

TABLE IV

*Correspondence between Total Base and Individual Cations in Human Blood Plasma (Heparinized)*

The values are given in milli-equivalents per liter. Total base was determined by the electrodialysis method on samples of 0.2 cc. The methods for individual cations are as given in Peters and Van Slyke (1932) (Na (p. 736), K (p. 741), Ca (p. 767), Mg (p. 785)).

Subject	Total base	Na <sup>+</sup>	K <sup>+</sup>	Ca <sup>++</sup>	Mg <sup>++</sup>	Difference
K	158.0	141.3	6.24	5.13	2.5	2.8
B	153.8	139.5	4.39	5.13	2.5	2.3
R	157.0	141.5	6.02	6.04	3	0.5
N	158.3	142.6	6.98	5.38	2.5	1.2
X	155.3	141.8	4.8	4.4	2.9	1.4
W†	168.8	154.1	5.97	6.15	3	-0.4
"†	157.6	142.4	4.41	5.82	3	2.1
"†	156.8	140.7	5.97	5.04	2	2.9
"†	151.4	138.3	5.06	5.42	2	1.0

\* The values for Mg are approximate except for Subject X.

† Samples during recovery from a work experiment in which extreme exhaustion was produced in slightly more than 1 minute.

TABLE V

*Bases Other Than Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>++</sup>, Mg<sup>++</sup> in Blood Plasma or Serum*

The values are given in milli-equivalents per liter. The valency is uncertain but is here assumed to be as given in the table.-

Base	Concentration	Authority
NH <sub>4</sub> <sup>+</sup> (preformed)	0.1 : 0.2	See above
Fe <sup>++</sup>	0.04:0.08	Tompsett (1934) p. 1536
Cu <sup>++</sup>	0.06:0.07	" (1934) p. 1544
Al <sup>+++</sup>	0.2	Wührer (1933)
Zn	Trace	Boyd and De (1933)
Su	"	" " "
Ag	"	" " "
Rb	"	" " "
Pb <sup>++</sup>	0.001:0.003	Litzner and Weyrauch (1933)
Mn <sup>++</sup>	0.004:0.01	Urechia, Pamfil, and Retezeanu (1934)

from the cells (Parnas and Heller, 1924), so it is advisable to separate plasma or serum intended for this analysis soon after drawing.

In ashed materials the ammonium ions are, of course, lost. The dilute  $\text{H}_2\text{SO}_4$  in the lower anode vessel must be renewed occasionally; if 20 cc. of 2 N acid are used, it should be renewed about every ten or fifteen determinations.

The analysis of cells is apt to be troublesome. Accuracy in pipetting 0.2 cc. is difficult with an ordinary pipette, but this is remedied by using a syringe-pipette (Krogh and Keys, 1931). The syringe-pipette, adjusted to deliver 0.2 cc. of distilled water, is flushed so that the dead space is filled with distilled water. The syringe is filled slowly with the cells and discharged into the cathode vessel. The syringe-pipette is then filled with distilled water about five times, the delivery being added in each case to the

TABLE VI

*Correspondence between Total Base and Individual Cations in Human Red Cells from Heparinized Blood*

Total base was determined by the electrodialysis method on 0.2 cc. samples. The methods for Na and K are as given by Peters and Van Slyke ((1932) p. 736, p. 751). Blood was obtained under various experimental conditions. The values are given in milli-equivalents per liter.

Subject No.	Total base	Na	K	Difference $\Sigma B - \Sigma (Na + K)$
1	116.2	15.2	103.0	-2.0
2	109.2	14.4	94.4	0.4
3	109.5	13.9	95.3	0.3
4	116.9	16.7	100.4	-0.2

cathode vessel. This whole operation is easy and rapid and the last washings will be entirely clear, the error being not more than 0.2 per cent. The high concentration of protein in the sample may cause clogging of the membrane surface or base may be entrapped in the protein coagula resulting during dialysis. These troubles are much lessened if the sample is suspended in a relatively large volume of water in the cathode vessel; 15 or 20 cc. will do for 0.2 cc. samples.

## SUMMARY

The electrodialysis method of Adair and Keys for total base gives results accurate to within 1 per cent when samples of 0.2 cc.

of biological fluids are used. By this method at least twenty-four determinations may be made within the course of the day.

Details of apparatus, procedures, and results are given.<sup>1</sup>

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<sup>1</sup> Since this paper was written this method has been applied to routine analyses of over 200 samples of plasma and over 50 samples of cells. In all cases 0.2 cc. samples, delivered from a Krogh-Keys pipette, were used. Aside from very occasional erratic results, the agreement between duplicates has been well within the limits reported in this paper. In about 50 cases the individual cations were also determined; the agreement between the sum of these and the total base was similar to that shown in Tables IV and VI.



## THE METABOLISM OF GLYCOLIC ACID IN PROGRESSIVE MUSCULAR DYSTROPHY

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Data on the question of whether glycolic acid can serve as a precursor of glycine in the body have been furnished by workers employing two different methods of investigation. Griffith (1) found that in rats given toxic quantities of sodium benzoate in addition to an adequate diet, the toxicity of the benzoic acid was decreased as effectively by glycolic acid as by glycine. No determinations of the benzoic acid derivatives in the urine of his animals are reported, although much earlier Griffith and Lewis (2) showed that glycolic acid does not increase the rate of synthesis of hippuric acid when administered with benzoate. Baer and Blum (3) and Greenwald (4) administered glycolic acid to diabetic animals and observed no increase in the excretion of urinary sugar. Although these workers were not primarily interested in the question of a possible conversion of glycolic acid into glycine, their results are of significance to this problem. It is well known that glycine given to phlorhizinized dogs will increase the urinary D:N ratio (5). Since the glycolic acid was without effect upon the excretion of sugar, it is improbable that any appreciable amount could have been converted into glycine.

In the present investigation the effect of glycolic acid upon the excretion of creatine in patients with progressive muscular dystrophy was studied. It has been shown by Brand, Harris, Sandberg, and Ringer (6), Thomas, Milhorat, and Techner (7), and others that the administration of glycine in progressive muscular dystrophy is followed by an increased excretion of urinary creatine. If glycolic acid can be converted into glycine in the body,



then its ingestion by these patients should increase the output of creatine in a manner similar to that following the ingestion of glycine. However, an increase in the creatinuria would not constitute definite proof of such a conversion, as the glycolic acid might have been synthesized to creatine without necessarily passing through the glycine stage, or it might have stimulated the creatine mechanism without having been converted into creatine. On the other hand, the absence of any effect of glycolic acid on the excretion of creatine would constitute evidence that the glycolic acid had not been converted into glycine in any appreciable amounts.

The possibility that the CHOH grouping in glycolic acid might be oxidized to the carboxyl radical in the organism was also investigated. Dakin (8) has shown that following the administration of glycolic acid in dogs there is a slight increase in the excretion of oxalic acid, although Pohl (9) could find neither glycolic acid nor oxalic acid in the urine of a dog given glycolic acid. This increase in output probably does not represent the absolute amounts of oxalic acid arising from the glycolic acid, as Dakin (8) has demonstrated that the body is capable of destroying small amounts of oxalic acid. In the present study the effect of glycolic acid on the output of oxalic acid was likewise determined.

#### EXPERIMENTAL

##### *Methods*

The subjects were patients with progressive muscular dystrophy in whom the ingestion of glycine was followed by a definite increase in the creatinuria, and in whom the ability to retain exogenous creatine was considerably impaired. The patients were kept in a special metabolism ward which permitted the quantitative collection of all specimens. The diet, which was creatine-creatinine-free, was supervised rigorously and kept constant for each experimental period. The dietary protein was so adjusted that the nitrogen intake for the glycine and glycolic periods was kept unchanged. This was done in order to permit a comparison between the effects of glycolic acid and glycine, as differences in the nitrogen intake can in themselves affect the level of the creatinuria.

Following an adequate control period, the patients were given glycolic acid, neutralized with sodium hydroxide. After a second

TABLE I

*Effect of Glycolic Acid on Creatinuria of Patients with Progressive Muscular Dystrophy*

Patient	Length of period	Diet	N intake	Average daily urinary output			Remarks
				Total N	Performed creatine	Creatine as creatinine	
	days	gm.	gm.	gm.	gm.	gm.	
C. M., 44.3 kilos	4	Protein 60, fat 60, carbohydrate 180	9.60	6.61	0.171	0.679	
	4	Same	9.60	7.07	0.179	0.729	Glycolic acid 10 gm. daily, 20 gm. on 1st day of period
	2	"	9.60	7.22	0.161	0.702	
	5	Protein 50, fat 60, carbohydrate 180	8.00	6.80	0.175	0.702	
L. V., 52.0 kilos	5	Same	9.80	8.06	0.168	0.772	Glycine 10 gm. daily
	5	"	11.70	13.17	0.161	0.950	
	4	Protein 60, fat 85, carbohydrate 200	11.40	8.16	0.259	0.717	" 20 " "
	4	Protein 49, fat 85, carbohydrate 200	9.60	7.65	0.251	0.670	" 10 " "
	5	Protein 60, fat 85, carbohydrate 200	9.60	6.62	0.264	0.572	
	6	Same	9.60	6.12	0.256	0.627	Glycolic acid 10 gm. daily, 20 gm. on 2nd day
	6	"	9.60	6.64	0.254	0.571	
	4	Protein 70, fat 90, carbohydrate 200	11.20	8.11	0.685	0.263	

TABLE I—*Concluded*

Patient	Length of period	Diet	N intake	Average daily urinary output			Remarks
				Total N	Preformed creatine	Creatine as creatinine	
	days	gm.	gm.	gm.	gm.	gm.	
M. S.,— <i>Concluded</i>	5	Protein 50, fat 90, carbohydrate 200	8.00	6.45	0.675	0.255	
	4	Same	9.80	7.84	0.664	0.360	Glycine 10 gm. daily
	7	"	11.60	9.46	0.651	0.434	" 20 " "
	2	Protein 70, fat 90, carbohydrate 200	11.20	8.47	0.648	0.232	
	4	Same	11.20	8.85	0.627	0.299	Glycolic acid 20 gm. daily
	4	"	11.20	8.38	0.632	0.193	
	2	"	11.20	7.82	0.633	0.274	Glycolic acid 20 gm. daily

control period glycine in equivalent amounts was administered. Since the molecular weights of the two substances are practically the same, the amounts administered represent molecular equivalents of the two substances. The urine was collected in exactly 24 hour periods and diluted to the same volume each day for the chemical determinations. For the determination of the creatine and preformed creatinine, the Benedict and Folin methods, respectively, were used. The oxalic acid was estimated by Dakin's modification of the Salkowski-Autenrieth and Barth method. The total urinary nitrogen was determined by the usual macro-Kjeldahl technique.

## DISCUSSION

The results are shown in Tables I and II. In all experiments there was an increase in the output of creatine when glycolic acid was given, although the effect was considerably less than that produced by equivalent amounts of glycine. When 20 gm. of glycolic acid were given, the average increase in the urinary creatine

was 0.057 gm. in the following 24 hour period. Equivalent amounts of glycine increased the creatine output 0.188 gm. The average rise in the creatine output after 10 gm. of glycolic acid

TABLE II

*Effect of Glycolic Acid on Creatinuria in a Patient with Progressive Muscular Dystrophy*

Patient A. S., 54 kilos.

Length of period	Diet	N intake	Average daily urinary output			Remarks
			Total N	Pre-formed creatinine	Creatine as creatinine	
days	gm.	gm.	gm.	gm.	gm.	
3	Protein 65, fat 75, carbohydrate 180	10.40	7.44	0.192	0.686	
2	Same	10.80	7.52	0.208	1.226	Creatine 1.32 gm. per os on 1st day of period; no retention
4	"	10.40	7.57	0.199	0.699	
4	"	10.40	7.69	0.206	0.755	Glycolic acid 20 gm. daily
9	"	10.40	7.60	0.202	0.688	
5	"	10.40	7.39	0.206	0.718	Glycolic acid 20 gm. daily
10	"	10.40	7.31	0.180	0.689	
4	"	11.30	8.59	0.192	0.708	Glycine 5 gm. daily
7	"	11.90	8.42	0.188	0.765	" 8 " "
5	"	10.40	7.34	0.187	0.690	
5	"	14.10	10.40	0.180	0.858	" 20 " "
3	"	10.40	7.40	0.183	0.703	
5	Protein 45, fat 75, carbohydrate 180	7.20	5.56	0.193	0.672	
5	Same	10.90	8.65	0.199	0.827	" 20 " "
8	"	7.20	5.19	0.199	0.684	
2	"	7.60	5.03	0.201	1.121	Creatine 1.32 gm. per os on 1st day; 14% retention
2	"	7.20	4.96	0.198	0.671	

was 0.052 gm., whereas the same amount of glycine was followed by an average increase of 0.116 gm. in urinary creatine. In one subject (Table II) given varying amounts of glycine, the effect of

20 gm. of glycolic acid was greater than 5 gm. of glycine and less than 8 gm. of glycine.

The output of oxalic acid in the urine was determined in two subjects (M. S. and A. S.). During three control periods of 2 to 4 days each, the average daily excretion of oxalic acid was 0.001 to 0.0015 gm. No increase in the output of oxalic acid was observed when 20 gm. of glycolic acid were given daily for three periods of 4 days each.

The results suggest the possibility that glycolic acid can be converted into glycine in the organism, although the amounts transformed into the amino acid appear to be only a fraction of the amounts administered. On the other hand, the synthesis of creatine from glycolic acid without the intermediate formation of glycine or a mere stimulatory effect of the glycolic acid on the creatine mechanism must be considered as possible, but perhaps less likely, explanations for the small increases in the creatinuria. Either no oxalic acid was formed by the oxidation of the glycolic acid or the amounts produced were less than those that the body is capable of destroying.

#### SUMMARY

The effect of glycolic acid on the creatinuria of patients with progressive muscular dystrophy was studied.

The ingestion of glycolic acid was followed by a small increase in the output of creatine which was considerably less than that produced by equivalent amounts of glycine.

The administration of glycolic acid was without effect on the urinary output of oxalic acid.

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## THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

### XLVI. PHTHIOCEROL, A NEW ALCOHOL FROM THE WAX OF THE HUMAN TUBERCLE BACILLUS\*

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The occurrence of two new alcohols, *d*-eicosanol-2 and *d*-octadecanol-2, in the unsaponifiable matter of the timothy bacillus wax (1) and in leprosin (2), a neutral wax-like substance obtained from the leprosy bacillus, has been reported recently from this Laboratory. The same alcohols have also been isolated from the wax of the avian tubercle bacillus (3).

In view of the wide distribution of these alcohols in acid-fast bacterial waxes, we have searched for them also in certain wax fractions derived from the human tubercle bacillus, especially in those fractions having properties similar to leprosin, but neither of these alcohols could be found. We found, however, another new alcohol which differs entirely in composition and properties from the alcohols referred to above. The same alcohol was first isolated in this Laboratory in 1928 during the investigations of the "purified wax" (4) and of the "soft wax" (5), but at that time the substance was not described or studied. This new alcohol appears to be one of the characteristic components of the waxes of the human tubercle bacillus, and, in order to indicate its distinctive character and origin, we propose to designate it by the name phthiocerol.

Phthiocerol is optically active,  $[\alpha]_D = -4.8^\circ$ , m.p. 73–74°, and

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† Holder of a National Tuberculosis Association Fellowship at Yale University, 1934–36.

its composition corresponds to the formula  $C_{35}H_{72}O_3$  or possibly  $C_{36}H_{74}O_3$ . The substance contains two hydroxyl groups and one methoxyl group.

A dihydric alcohol called "phytogycol," a cleavage product of the wax from the human tubercle bacillus, has been described by Stendal (6). The properties of "phytogycol" ( $[\alpha]_D = -4.2^\circ$ , m.p.  $73^\circ$ , and two active hydrogen atoms) are similar to those which we have observed in the case of phthiocerol; in fact the two substances are probably identical. On the basis of analyses of phytogycol and its derivatives, Stendal assigned to it the formula  $C_{26}H_{54}O_2$ .

The analytical values for phthiocerol differ somewhat from those reported by Stendal for "phytogycol," and moreover we found by the Zeisel method that a quantity of methyl iodide was liberated which corresponded to one methoxy group in a compound having the formula  $C_{35}H_{72}O_3$  or  $C_{36}H_{74}O_3$ . The physiological properties of phthiocerol have not yet been determined.

#### EXPERIMENTAL

The so called "purified wax" from the human tubercle bacillus, prepared as described in a former paper (4), is not a homogeneous compound but a mixture. When the material is treated with hot acetone or with a mixture of ether and acetone, it is possible to remove a small amount of a white wax-like substance having a comparatively low melting point.

The substance used in the present investigation was obtained by treating the "purified wax" with hot acetone and with a mixture of ether and acetone until the extracts on evaporation to dryness left practically no residue. The wax-like substance which remained on evaporation of the extracts was readily soluble in ether and dissolved slowly in hot acetone, but it was practically insoluble in cold acetone or alcohol. For purification the substance was dissolved in ether, an equal volume of acetone was added, and the solution was cooled in ice water. A heavy granular precipitate separated which, after being filtered, washed with acetone, and dried, formed a snow-white powder, m.p.  $55-56^\circ$ ,  $[\alpha]_D = +0.55^\circ$  in  $CHCl_3$ . It was free from nitrogen and contained only the merest trace of phosphorus. About 55 gm. of substance were available for the present study.

**Saponification**—On account of the very slight solubility of the wax in alcohol the saponification was carried out in benzene-methyl alcohol solution. 53.8 gm. of substance were dissolved in 300 cc. of benzene to which 300 cc. of methyl alcohol containing 15 gm. of potassium hydroxide were added. The wax is extraordinarily difficult to saponify and it was necessary to reflux the solution for 145 hours before all of the neutral or unsaponifiable matter was liberated.

The alkaline solution was mixed with ether, transferred to a separatory funnel, and washed thoroughly with dilute hydrochloric acid, after which it was washed with water until the washings were neutral. The solution was dried over sodium sulfate, filtered, and evaporated to dryness. The residue was dissolved in ether and precipitated by the addition of alcohol; after filtration the ether was removed by distillation, and the precipitate which separated on cooling was removed and combined with the first precipitate. The alcohol-insoluble products thus obtained resembled in properties the so called "unsaponifiable wax" (4) and were reserved for a future investigation.

In order to remove any additional higher fatty acids from the alcoholic solution, an excess of lead acetate dissolved in hot alcohol was added and the precipitate which separated on cooling was filtered off and washed with alcohol. The filtrate was concentrated *in vacuo* nearly to dryness, after which the residue was mixed with water and extracted with ether. The ethereal solution was washed with water, dried over sodium sulfate, filtered, and evaporated to dryness. The residue, consisting of crude phthiocerol, weighed 4.4 gm. and was recrystallized from ethyl acetate or acetone until the constant melting point of 73–74° was attained. The substance crystallized from ethyl acetate in rosettes of prismatic needles. The substance did not absorb any bromine and gave no coloration in the Liebermann-Burchard reaction.

<i>Analysis</i> — $C_{35}H_{72}O_3$ (540).	Calculated.	C 77.78, H 13.33
$C_{36}H_{74}O_3$ (554).	"	" 77.98, " 13.35
	Found.	" 77.64, 77.84, 77.76, H 13.29, 13.55, 13.49

**Molecular Weight**—1.82 mg. substance in 9.15 mg. camphor gave a depression of 15.1°. Found, mol. wt. 527

**Rotation**—0.4134 gm. substance dissolved in chloroform and diluted to 10 cc. gave in a 1 dm. tube an average reading of  $-0.20^\circ$ ; hence  $[\alpha]_D^{25} = -4.8^\circ$



*Phthiocerol Isolated in 1928*—In the purification of the so called "unsaponifiable wax" (4), a fraction was isolated from the mother liquors which, after several crystallizations from acetone, melted at 73–74°. This fraction has now been examined more closely. On recrystallization from ethyl acetate it separated in rosettes of colorless needles. The substance which melted at 73–74°,  $[\alpha]_D^{23} = -5.0^\circ$ , was identical in every respect with the recently isolated phthiocerol.

In the analysis of the so called "soft wax" ((5) p. 332) a neutral fraction was isolated. This material has been examined recently; it was possible to isolate from it by crystallization from ethyl acetate 0.12 gm. of pure phthiocerol, m.p. 73–74°.

*Acetylation of Phthiocerol*—When phthiocerol is acetylated by the method of Verley and Bölsing (7), values are obtained corresponding to two acetyl groups.

0.1987 gm., 0.02411 gm., 0.02546 gm. substance took up 0.03152 gm., 0.00381 gm., 0.00400 gm.  $\text{CH}_3\text{CO}$

$\text{C}_{38}\text{H}_{72}\text{O}_3$  (540). Calculated.  $2\text{CH}_3\text{CO}$  15.92

$\text{C}_{36}\text{H}_{74}\text{O}_3$  (554). " " 15.52

Found.  $\text{CH}_3\text{CO}$  15.86, 15.80, 15.71

*Phthioceryl Acetate*—The acetylation products obtained in the reactions mentioned above were collected and purified by precipitation from alcohol by cooling. A white powder was obtained which was not definitely crystalline. The substance melted at 37–38°, solidified at 31.5°, and remelted at 34°.

*Analysis*— $\text{C}_{38}\text{H}_{70}\text{O}_3(\text{COCH}_3)_2$  (624). Calculated. C 75.00, H 12.18

$\text{C}_{36}\text{H}_{72}\text{O}_3(\text{COCH}_3)_2$  (638). " " 75.23, " 12.22

Found. " 75.32, 75.36, H 12.34, 12.34

*Molecular Weight*—2.33 mg. substance dissolved in 10.39 mg. camphor gave a depression of 15.4°. Found, mol. wt. 582

Acetylation in pyridine solution with acetic anhydride at room temperature gave derivatives which could not be obtained in crystalline form. They were extremely soluble in the ordinary solvents and readily soluble in alcohol but less so in methyl alcohol. When precipitated from alcohol or methyl alcohol by cooling, amorphous powders separated which, after being filtered, washed, and dried, formed a wax-like mass, m.p. 30°. 0.2846 gm. of the

acetyl derivative on saponification required 15.45 cc. of 0.05946 N NaOH.

<i>Analysis</i> — $C_{26}H_{70}O_3(COCH_3)_2$ (624).	Calculated.	$CH_3CO$	13.78
$C_{26}H_{72}O_3(COCH_3)_2$ (638).	"	"	13.47
	Found.	"	13.87

The analytical result is in agreement with the calculated value for two acetyl groups.

Determination of methoxyl by the Zeisel method gave the following results.

<i>Analysis</i> — $C_{26}H_{72}O_3$ (540).	Calculated.	$OCH_3$	5.74
$C_{26}H_{74}O_3$ (554).	"	"	5.59
	Found.	"	5.30

*Characterization of the Alkoxy Group*—The volatile iodide liberated on heating 72.5 mg. of phthiocerol with hydriodic acid and phenol was led into an alcoholic solution of trimethylamine and gave 16.5 mg. of white crystals. Evaporation of the filtrate to dryness left a residue weighing 9 mg. The total yield of tetramethylammonium iodide was therefore 25.5 mg., which represents 98 per cent of the theoretical amount of methyl iodide. The crystalline substance gave on analysis values in agreement with the calculated composition of tetramethylammonium iodide.

<i>Analysis</i> — $(CH_3)_4NI$ (200.92).	Calculated.	C	23.89,	H	5.97
	Found.	"	24.12,	"	5.94

*Hydrocarbon*—The iodide resulting from the methoxy determinations was isolated and reduced by heating in acetic acid solution with amalgamated zinc. The hydrocarbon was purified by recrystallization from ethyl acetate. The substance melted at 58.5–59.5°, solidified at 58.5–58°, and remelted at 58.5–59.5°.

<i>Analysis</i> — $C_{24}H_{70}$ (478).	Calculated.	C	85.35,	H	14.64
	Found.	"	84.93,	"	14.59

Further experimental work is under way dealing with the chemical structure of phthiocerol.

#### SUMMARY

Phthiocerol, a higher optically active alcohol and a characteristic constituent of the wax of the human tubercle bacillus, has been

isolated and some of its properties and derivatives have been described.

From the present available analytical data it is impossible to decide whether the formula of phthiocerol is  $C_{34}H_{67}(OH)_2OCH_3$  or  $C_{35}H_{69}(OH)_2OCH_3$ , because the difference in the calculated composition of the two is less than the experimental error. The analytical values obtained would fit either formula.

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**ON THE MECHANISM OF ENZYME ACTION**  
**A STUDY OF THE DECOMPOSITION OF MONOETHYL HYDROGEN**  
**PEROXIDE BY CATALASE AND OF AN INTERMEDIATE**  
**ENZYME-SUBSTRATE COMPOUND**

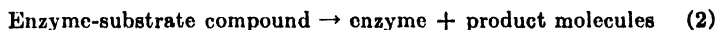
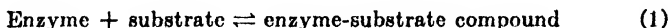
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(Received for publication, March 23, 1936)

The classical methods of enzyme chemistry were of necessity restricted to the study of the catalysis as a whole. Either the rate of disappearance of the substrate or the rate of formation of the end-products was measured. The kinetics of these processes could in some instances be explained by the assumption that the catalyst combines with the substrate to form an unstable intermediate which may either reversibly dissociate into the two components or break down with the formation of free enzyme and the split-products (1, 2).

The spectroscopic observation of the formation and the breakdown of an intermediary enzyme-substrate compound during the action of catalase on monoethyl hydrogen peroxide (3) permits an experimental analysis of the two main phases of the enzyme reaction:



Reaction 1 lends itself to optical study. The over-all process (Reactions 1 + 2) is measured by volumetric determination of the substituted peroxide.

**EXPERIMENTAL**

*Preparation of Enzyme Solutions*—Purified catalase solutions were prepared from horse liver<sup>1</sup> essentially according to Zeile and

<sup>1</sup> The author is greatly indebted to Chappel Brothers, Inc., Rockford, Illinois, for generously supplying the frozen horse liver used for these preparations.

Hellström (4). It was found expedient to carry out all operations of separation in a centrifuge which can accommodate four bottles of 500 cc. capacity each. Alumina gel (aluminum hydroxide, pure, moist (Eimer and Amend)) was used as adsorbent. Some data concerning the four enzyme solutions used in the present experiments are given in Table I.

For most of the experiments the undiluted enzyme preparations were used. These were dark brown in color and showed the typical catalase absorption spectrum in layers ranging from 2 to 5 cm., depending on their enzymatic activity. The secondary sodium phosphate used for the elution of the enzyme from the alumina gel adsorbate was neutralized by adding solid primary

TABLE I  
*Data for Catalase Preparations*

Catalase No	Liver used	Eluate obtained	Activity*
	<i>gm</i>	<i>cc.</i>	<i>k</i>
XXIX	4,500	500	7125
XXXIV	3,700	200	2020
XXXV	3,700	250	5300
XXXVI	10,000	400	4525

\* The activity was determined by measuring the rate of hydrogen peroxide decomposition by the highly diluted enzyme solution at 0°, pH 6.6, and at a total substrate concentration of 0.01 M. *k* is obtained as the product of the monomolecular velocity constant, calculated from the amount of substrate destroyed within the first 5 minutes of the experiment, and of the dilution factor of the enzyme.

potassium phosphate. The enzyme solutions were stored over chloroform in the refrigerator (approximately at +2°) and filtered before use.

*Preparation of Substrate Solutions.*—Solutions of monoethyl hydrogen peroxide were prepared by following essentially the directions given by Baeyer and Villiger (5) and by Ricche (6). The alkylation of hydrogen peroxide was found to give better yields if carried out with efficient cooling, the reaction temperature being kept near 10° by external cooling with an ice and water mixture. Throughout the alkylation process (requiring about 6 hours) and the subsequent neutralization with sulfuric acid, the reaction mixture was vigorously stirred with an electric stirrer.

The distillation of the substituted peroxide together with water was carried out under atmospheric pressure. The temperature inside the distillation flask was near 100°, whereas the oil bath was heated up to 150°. The distillation was discontinued after approximately one-half of the reaction mixture had been distilled over.

Since a sample of pure monoethyl hydrogen peroxide prepared according to the ether extraction method of Rieche and Hitz (7) gave essentially the same results as the dilute solutions obtained by single distillation, the distillates obtained as described above were used directly for the experiments after neutralization with solid secondary sodium phosphate. The amount of peroxide was determined by the iodometric method outlined below and was found to be between 1 and 2 M. These solutions were found to keep well for several months if stored in the refrigerator.

*Volumetric Study of Decomposition of Monoethyl Hydrogen Peroxide by Catalase*

*Method*

For the estimation of monoethyl hydrogen peroxide Baeyer made use of an iodometric method. According to Rieche (8), however, the active oxygen of this peroxide may not be quantitatively determined with either hydriodic acid or titanous trichloride. The latter author therefore carried out his analysis by oxidation with chromic acid; the acetic acid formed was distilled off in the presence of an excess of phosphoric acid and determined by acidimetric titration. This procedure is not only somewhat tedious but is also not applicable to solutions containing foreign organic matter. Inasmuch as the object of most of the experiments reported in this paper was to follow the breakdown of the substituted peroxide as catalyzed by the enzyme and to study the effect of temperature, pH, etc., on the rate of this reaction, it was not necessary to assay accurately the absolute concentration of the peroxide but only to record the relative changes in concentration with time. It was found that the iodometric method as used by the author for studying the breakdown of hydrogen peroxide by catalase (9) was adequate for this purpose. The oxidation of hydriodic acid by the substituted peroxide is slower than by hydrogen peroxide even in the presence of molybdic acid. Except

in the lowest concentrations of the peroxide used, it was found sufficient to wait for 1 hour between the addition of the peroxide to the acid potassium iodide solution plus a few drops of molybdic acid solution and the titration with thiosulfate with starch as internal indicator.

The relationship between the amount of iodine liberated and the concentration of monoethyl hydrogen peroxide was established in the following manner. In ten flasks a 1.1 M peroxide solution was introduced in amounts varying from 0.1 to 1.0 cc. and made up to 5 cc. by addition of water. These solutions were allowed to react

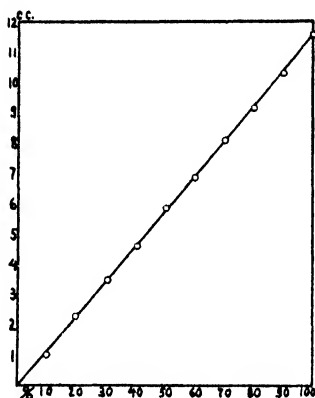


FIG. 1. Curve illustrating the proportionality between the amounts of ethyl hydrogen peroxide present and the amounts of 0.1 N thiosulfate required. The peroxide concentration (abscissa) is given in per cent of the maximum concentration used (0.2 M).

for 1 hour with 5 cc. of a 10 per cent potassium iodide solution plus 2 cc. of 33 per cent sulfuric acid plus 3 drops of a saturated aqueous solution of molybdic acid. The titration was carried out with 0.1 N thiosulfate solution. The determination was performed in duplicate. The curve in Fig. 1 was drawn through points representing the averages of the two individual values obtained. From the diagram it follows that a strictly linear relationship exists under the conditions of the experiment. Furthermore, it is to be concluded that if the reaction between the peroxide and hydriodic acid is not going to completion, the percentage deviation from the theoretical value remains constant in

the concentration range tested. The method could therefore be used with confidence for the present purpose.

*Order of Reaction*—3 cc. of the undiluted Catalase XXXIV ( $k = 2020$ ) and 1 cc. of M/15 phosphate buffer (pH 6.79) were placed in a 50 cc. Pyrex beaker and mechanically stirred at a moderate rate. At the experimental time = 0, 1.0 cc. of 1.1 M monoethyl hydrogen peroxide was quickly added. The reaction was stopped

TABLE II

*Kinetics of Decomposition of Monoethyl Hydrogen Peroxide by Catalase at Different Temperatures*

Each titration figure given was obtained by a separate experiment.

Reaction stopped at time $t$	0.1 N thiosulfate required	0.1 N peroxide decomposed	$k$ (monomolecular)	$k$ (zero order)
At 23°; 2 cc. Catalase XXXIV ( $k = 2020$ ) + 2 cc. M/15 phosphate buffer (pH 6.79) + 1 cc. 1.1 N $C_2H_5OOH$				
min.	cc.	cc.	$\frac{1}{t} \log \frac{a}{a-x}$	$\frac{x}{t}$
0	10.27			
3	7.98	2.29	0.037	0.76
6	5.2	5.07	0.049	0.84
9	2.86	7.41	0.062	0.82
12	1.19	9.08	0.078	0.76
15	0.32	9.95	0.100	0.66
At 0°; 3 cc. Catalase XXXIV + 1 cc. M/15 phosphate buffer (pH 6.79) + 1 cc. 1.1 N $C_2H_5OOH$				
0	11.20			
3	9.25	1.95	0.028	1.95
6	8.38	2.82	0.021	1.41
12	6.53	4.67	0.020	1.17
24	3.44	7.76	0.021	0.97
48	1.04	10.16	0.022	0.64

at various intervals by addition of 3 cc. of 33 per cent sulfuric acid. The glass stirrer was lifted above the surface of the mixture and rinsed with a few cc. of distilled water. 4 cc. of 10 per cent potassium iodide solution and 3 drops of molybdic acid solution were added. After standing for 1 hour, the liberated iodine was titrated with 0.1 N thiosulfate. Experiments were conducted at room temperature (22°) and also at 0–1° (beaker immersed in a water-ice mixture). The solutions in the case of the latter series



were all previously cooled. The results are given in Table II. From them were calculated both the monomolecular and the zero order reaction constants, as shown in the last two columns of the table.

The calculations show that whereas the course of the reaction at the low temperature may satisfactorily be described by the equation of the first order, the data obtained at 22° yield greatly increasing monomolecular reaction constants in time and appear to fit approximately the equation of the linear relation. No explanation at present can be offered for this observation.

*Effect of Temperature on Rate of Reaction*—For these experiments a water thermostat was used, the temperature of which

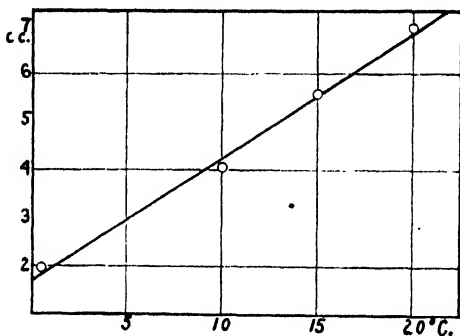


FIG. 2. Variation of the reaction velocity with the temperature. The ordinate represents the amounts of substrate decomposed in 5 minutes, expressed in cc. of 0.1 N thiosulfate.

could be adjusted to any desired value between  $+0.1^{\circ}$  and  $+99^{\circ}$  with a constancy of  $\pm 0.003^{\circ}$ . The reaction time was 5 minutes throughout. The value for time = 0 was obtained as the average of four determinations in which the sulfuric acid had been added before the substrate. The actual runs at the four temperatures selected were performed in quadruplicate, and the average obtained of the four values was used for the plotting of Fig. 2. From Fig. 2 it appears that there exists a linear relationship between temperature and rate of reaction as expressed by turnover of substrate.  $Q_{10}$  in the interval  $0-10^{\circ}$  equals 2.3 and between  $10-20^{\circ}$ , 2.19.

*Effect of Hydrogen Ion Concentration*—In these experiments

1.0 cc. of Catalase XXXV ( $k = 5300$ ) was added to 1.0 cc. of 1.1 M monoethyl hydrogen peroxide and 3.0 cc. of buffer mixtures ranging from pH 3.85 to 10.43. All the pH values given in this paper were obtained by measurements with the glass electrode in solutions of identical composition with that of the mixtures used for titration; these had been allowed to stand for at least 1 hour to allow the enzymatic reaction to go to completion. The author wishes to thank Mr. D. DuBois for performing the pH determinations with the modified glass electrode and circuit as devised by him.

The temperature in these experiments was kept near  $0^\circ$  by cooling with ice and water. Borate, phthalate, acetate, phosphate, and glycocoll buffer mixtures of a molar strength of 0.06

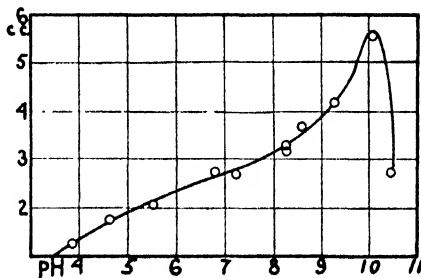


FIG. 3. Activity-pH curve. The ordinate represents the amounts of peroxide decomposed in 10 minutes, expressed in cc. of 0.1 N thiosulfate.

to 1.0 were used. No attempt was made to carry out all experiments at the same ionic strength, as a variation of this value did not appear to affect the reaction rate appreciably. All experiments were done in duplicate. The resulting pH-activity curve is given in Fig. 3. Whereas for hydrogen peroxide as substrate catalase will show an activity optimum between pH 6.5 and 9, slightly varying according to the source of enzyme material used and to the different investigators (10), this curve shows that the activity towards the substituted peroxide rises steadily with pH, attaining a sharp maximum near 10 and falling sharply again beyond this value, probably due to enzyme destruction at this hydrogen ion concentration. Control experiments showed that the rather alkaline optimum is not an artifact due to an instability of the peroxide at this pH. In spite of this finding, most of the

present experiments were conducted near pH 7 in order to exclude the possibility of any secondary reactions caused by the high hydroxyl ion concentration and in order to facilitate a comparison with the enzymatic decomposition of hydrogen peroxide which is commonly studied at a pH slightly below 7.

*Affinity of Catalase for Monoethyl Hydrogen Peroxide*—The so called Michaelis constant ( $K_m$ ), *i.e.* the substrate concentration at which the enzymatic reaction proceeds at half the maximal speed, was determined in a manner analogous to former experiments in which hydrogen peroxide was the substrate (11). The fact that monoethyl hydrogen peroxide has some affinity for

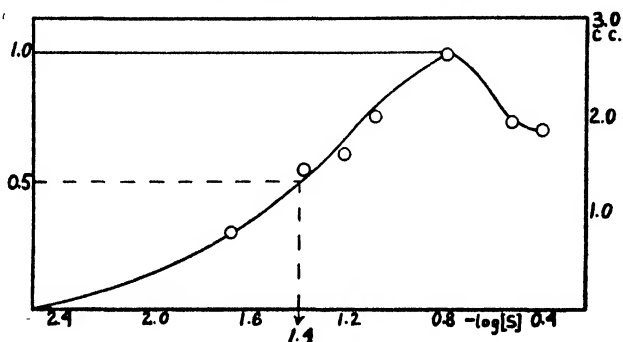


FIG. 4. Activity- $P_{[S]}$  curve. The abscissa represents the negative logarithm of the substrate concentration; the right ordinate, amounts of peroxide decomposed in 5 minutes, expressed in cc. of 0.1 N thiosulfate; the left ordinate, the rational measure, the maximal reaction rate being taken as 1.0.

catalase was borne out in the earlier work, when it could be shown that the inhibition of the catalase-hydrogen peroxide reaction by the substituted peroxide is of the competitive type.

Of the five series of experiments carried out with constant enzyme concentration and varying substrate concentration, the most satisfactory one was used for plotting the curve in Fig. 4. The reaction mixtures consisted of 3.0 cc. of M/15 phosphate buffer of pH 6.79, amounts of a 2.07 N monoethyl hydrogen peroxide solution (neutralized) varying from 0.05 to 1.0 cc., 1.0 cc. of Catalase XXXV ( $k = 5300$ ), and distilled water to make up a total volume of 5.0 cc. The reaction time in this series was 5 minutes and the temperature near 0°. The curve shows the variation of

substrate turnover under these conditions with a variation of the total substrate concentration between 0.0207 and 0.414 N. As usual, not the absolute substrate concentration but the negative logarithm of this value is taken as the abscissa. This manner of plotting has the advantage that the experimental points corresponding to low substrate concentration are more evenly spaced than they would be by direct use of the concentration values. The Michaelis constant, which is the parameter of the left upward branch of the activity- $P_{[S]}$  curve, is found to be nearly  $-\log [S] = 1.4$ . The corresponding value for  $[S]$  is 0.04 N. The  $K_m$  value for hydrogen peroxide as a substrate was formerly found to be 0.066 N = 0.033 M. Since in the case of a mono-substituted peroxide the normality equals molarity, it follows that the affinity of catalase for both substrates is very similar.

The descending branch of the activity- $P_{[S]}$  curve at higher substrate concentrations is similar to that of the curves with hydrogen peroxide as substrate (11). It is a demonstration of the fact that at high substrate concentrations the enzyme is inhibited. For ethyl hydrogen peroxide sufficient data are not yet available to determine with accuracy the value of the parameter of the descending branch of the activity- $P_{[S]}$  curve. A graphical extrapolation, however, would yield a value for this "second Michaelis constant" of the order of magnitude found for hydrogen peroxide (0.4 M).

*Thermolability of Catalyst*—In order to ascertain that no thermostable non-enzymatic factor present in the enzyme preparation is catalyzing the breakdown of the substituted peroxide, 5 cc. of Catalase XXXV ( $k = 5300$ ) were pipetted in a test-tube and heated for 10 minutes in a boiling water bath. A dark brown clot formed during this procedure and was filtered off. 1 cc. of this solution when added to 3 cc. of phosphate buffer, pH 6.79, and 1 cc. of 1.1 N monoethyl hydrogen peroxide solution, did not cause a decrease of the peroxide titer within 10 minutes at 0°. In a control experiment with non-treated enzyme solution an amount of peroxide equivalent to 2 cc. of 0.1 N thiosulfate was decomposed. The catalysis is therefore due to a thermolabile agent.

*Inhibition by Cyanide*—In a mixture consisting of 1 cc. of 1.1 N monoethyl hydrogen peroxide, 3.9 cc. of phosphate buffer, pH 6.79, 1 cc. of Catalase XXXV ( $k = 5300$ ), and 0.1 cc. of 0.1 M

sodium cyanide (neutral), no cleavage of the peroxide was observed within 10 minutes at 0°. The complete inhibition of the catalysis by  $2 \times 10^{-3}$  M HCN suggests that a catalyst containing trivalent iron is concerned in the reaction. Catalase has been shown to contain stabilized ferric iron combined with protoporphyrin as the prosthetic group of the enzyme (12). The reaction between catalase and hydrogen peroxide is already completely inhibited by  $2 \times 10^{-4}$  M HCN (11).

*Reaction Products*—Whereas hydrogen peroxide, when acted upon by catalase, yields molecular oxygen and water as the final reaction products, in the breakdown of monoethyl hydrogen peroxide no appreciable amounts of gas are released. Instead, a strong odor of acetaldehyde is noticed. The formation of aldehyde was qualitatively demonstrated by the well known condensation test in the presence of strong alkali and by the blue color produced by addition of sodium nitroprusside and piperidine. It should be mentioned, however, that even the untreated peroxide solution gave a somewhat positive aldehyde test.

In order to test for the possible formation of free acid, *e.g.* acetic acid, in the course of the process, 3 cc. of Catalase XXXIV ( $k = 2020$ ) and 1 cc. of M/15 phosphate buffer, pH 6.79, were placed in a small crucible. An electric stirrer, a glass electrode, and a calomel electrode were inserted in the solution. The potential value was recorded, and 1.0 cc. of 1.1 N monoethyl hydrogen peroxide solution was added. The introduction of the buffered peroxide solution caused some shift in the pH. While the reaction proceeded, the potentials were recorded in short intervals. There was no detectable pH change within 5 minutes; therefore no acid is formed in the reaction.

According to Rieche (8), monoethyl hydrogen peroxide may break down in various ways, depending on the agent used; *e.g.*, on attack by alkali at higher temperatures only very little gas is formed, whereas under the action of formaldehyde and alkali much hydrogen and, moreover, formic acid, acetic acid, ethyl alcohol, and acetaldehyde are formed in a vigorous reaction.

It is planned to subject the enzymatic breakdown of the peroxide to further and quantitative analysis.

*Optical Study of Intermediate Enzyme-Substrate Compound. Technique*—The exact position of the absorption bands of the intermediate was determined with a Hilger wave-length spectrom-

eter. The accuracy of the setting of the wave-length drum was checked by the emission lines of an electrical sodium burner (Zeiss). For the other observations calibrated pocket spectroscopes (Brown, Zeiss) were used. Such straight vision instruments of small dispersion permit a better definition of absorption bands and an easier spotting of weak bands than big spectroscopes of the Kirchhoff-Bunsen type. Most of the observations were made visually, but the whole spectral cycle was also photographically recorded by means of a miniature roll film camera of wide

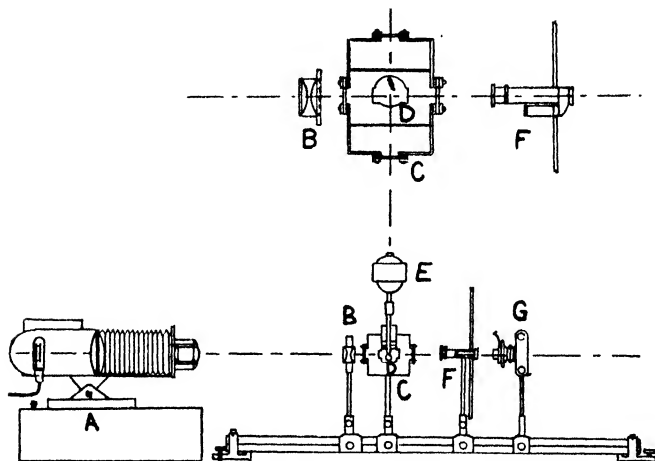


FIG. 5. Schematic representation of the arrangement used for optical study of the enzyme-substrate compound: A represents the projection lantern; B, condenser; C, trough for cooling; D, experimental vessel; E, motor and stirrer; F, pocket spectroscope; G, roll film camera.

aperture ( $f = 2.9$ ) and supersensitive panchromatic film (Eastman).

In those experiments where rapid mixing of enzyme and substrate was desired, a Pyrex tubularly-shaped cell with fused on windows and an inserted glass stirrer was used. The enzyme, buffer, and substrate solutions were stratified above each other by means of different concentrations of sucrose and the motor stirrer started at 0 time as suggested by Stadie (13). It was ascertained that sucrose in the concentrations used is no inhibitor

for the enzyme reaction. The details of the arrangement are shown in Fig. 5 which is largely self-explanatory.

*Qualitative Observations*—On direct visual observation in transmitted light and in the thickness of layer used for the spectroscopic experiments the enzyme solutions appear brown in color. Upon the addition of monoethyl hydrogen peroxide, there is a rapid color change to a greenish hue. Within the following

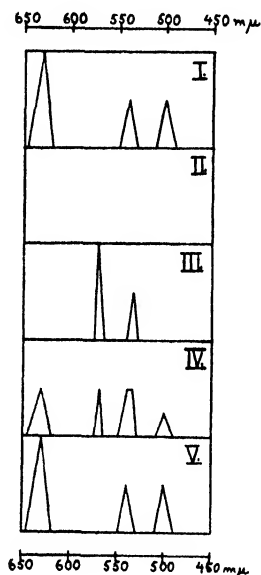


FIG. 6. Schematic representation of the spectroscopic cycle. *I* represents the spectrum of free enzyme; *II*, lag period during which a greenish color but no discrete absorption bands are noticed; *III*, spectrum of enzyme-substrate compound; *IV*, coexisting intermediate and free enzyme; *V*, restored enzyme spectrum. (After direct observation with the spectroscope. The heights of the bands indicate their visual intensity.)

seconds the red color of the intermediate catalase-peroxide compound appears. In the course of the breakdown of the compound which requires time of the order of minutes, the red tint fades and with the reformation of the free enzyme the original brown color is restored. The corresponding changes in light absorption, as observed with a small spectroscope, are represented schematically in Fig. 6. No specific absorption bands are visible during

the greenish transition period (II). It is therefore possible that this color shade is due neither to the enzyme nor to an intermediate but to concomitant pigments in the enzyme solutions (biliverdin, heptoflavin), the absorption bands of which are located in the far red and violet regions respectively.

The whole cyclic phenomenon may again be released by addition of fresh substrate. The restoration of the original enzyme spectrum is accompanied by the disappearance of titratable peroxide from the system.

*Position of Absorption Bands of Intermediate*—In order to prolong the visibility of the spectrum of the enzyme-substrate complex, the measurement was carried out at 0°. 5 cc. of Catalase XXIX ( $k = 7125$ ) and 5 cc. of a 0.41 N monoethyl hydrogen peroxide were cooled separately in ice. A small absorption tube of the Baly type with fused on windows and ground joints (Schott and Genossen, Jena) was also cooled. The solutions were mixed and transferred at once to the tube. The tube was submersed in an ice-filled trough. In the Hilger spectrometer, the long wave absorption band of the intermediate was better defined and more intense than the short wave band. Below are given the data obtained together with the values reported by Zeile and Hellström (4) for the uncombined enzyme, with which values our own observations were in satisfactory agreement.

*Spectrum of Free Enzyme—*

I. 650 . . . $\underbrace{646-620}_{629}$ . . . 610;	II. $\underbrace{550-530}_{540}$ . . . 520 . . . $\underbrace{510-490}_{500}$ $m\mu$
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*Spectrum of Enzyme-Substrate Compound—*

I. $\underbrace{576-564}_{570}$ ;	II. $\underbrace{540-529}_{534.5}$ $m\mu$
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*Effect of Temperature on Rate of Formation of Intermediate—*

The arrangement shown in Fig. 5 was used, with the exception of the film camera. The vessel with the electric stirrer in place was dipped into the trough filled with water of room temperature (22°). The bottom layer in the experimental vessel consisted of 15 cc. of a mixture of 10 parts of Catalase XXXVI ( $k = 4525$ ) and 5 parts of a sucrose solution prepared by dissolving 100 gm.



of pure sucrose in 100 cc. of water. A layer of 2.5 cc. of a mixture of 20 parts of *m*/15 phosphate buffer (pH 6.79) and 5 parts of the sucrose solution was carefully stratified above the bottom layer. The top or third layer was formed by 2.5 cc. of 2.07 *N* monoethyl hydrogen peroxide solution containing no sucrose. The electric stirrer was started at 0 time, and the interval lapsing before the stronger absorption band of the enzyme-substrate compound at 570 *mμ* could be first detected by visual observation through the pocket spectroscope was recorded. For three runs the time intervals 4.5, 3.6, and 3.4 seconds were obtained, averaging 3.8 seconds. In the following series the side compartments of the external trough were filled with ice. They were separated from the middle compartment which contained water, by means of metal gauze. Before beginning the experiment the vessel containing the three layers of solution was cooled for at least 15 minutes. The temperature of the ice water surrounding the vessel was 2°. Under these conditions the time intervals recorded in three trials were 6.3, 6.4, and 6.3 seconds, averaging 6.3 seconds. It follows that from 2–22° the rate of formation of the intermediate increases 1.6 times. Assuming a linear relationship as in the case of the over-all reaction,  $Q_{10}$  in this interval would be  $\sqrt{1.6} = 1.26$ . This value is smaller than  $Q_{10}$  for the over-all reaction (Table III). It is preferable not to base calculations of the apparent critical increment of the combination of the enzyme with the substrate on this result which was obtained with a primitive technique. It is probable, however, that the apparent activation energy of this reaction is somewhat smaller than that of the over-all reaction, and more specifically of the breakdown of the enzyme-substrate compound.

*Effect of Hydrogen Ion Concentration*—With the external trough filled with water of room temperature (22°), similar experiments were conducted with buffers of different pH. It was found that variation of pH between 4.11 and 8.82 is without a significant effect on the rate of the formation of the enzyme-substrate compound. Nine readings at four different hydrogen ion concentrations yielded an average time value of 4.0 seconds, the highest figure obtained being 5.3 and the lowest 3.4 seconds.

The pH values given were obtained by measurements with the glass electrode after the experiment.

*Experiments on Reality of Observed Lag Period*—The time necessary for complete mixing under the conditions of the preceding experiments was determined by measuring the length of time required for uniform distribution throughout the system of a top layer containing methylene blue dissolved in water. The bottom layer consisted of 10 cc. of water and 5 cc. of sucrose solution; the middle layer contained 2.5 cc. of a mixture of 20 parts of water with 5 parts of sucrose solution. Direct visual observation yielded a time value of 1.6 and 1.4 seconds, respectively. If the spectroscope was used, values of 2 seconds were obtained in two experiments. It follows that although the time necessary for complete mixing was comparatively long, the above rates observed for the appearance of the intermediate were distinctly slower than would be warranted by the mixing time. However, it appeared desirable to decrease the mixing time considerably so as to make it negligible compared with the observed time lag in the formation of the intermediate. Dr. W. C. Stadie, who had used a similar arrangement for the study of carbonic anhydrase (13), was good enough to advise the author on this aspect. Consequently, the rate of the stirring device was increased. Furthermore, the specific gravity of the different layers was lowered by the use of more dilute sucrose solutions. In order to avoid the foaming which occurred at the great speed of stirring, it was necessary but also sufficient to connect the motor with a Morse telegraph switch and to press the switch down for the length of time necessary to start the stop-watch. The sucrose content of the bottom layer in the final experiments was 5 per cent, in the middle layer 2.5 per cent, and in the top layer 0. By means of methylene blue control experiments, it was found that a stirring time of 0.2 to 0.3 second was sufficient to obtain uniform mixing. In three runs with the enzyme and substrate mixture at pH 6.6 and 22° the time lapsing before the first appearance of the stronger absorption band of the intermediate was found to be 1.5, 2.3, and 2.0 seconds, respectively. Since in these last experiments the mixing time was small compared with the observed lag period, some confidence may be placed in the reality of its existence.

*Amount of Substrate Required for Complete Transformation into Intermediate*—It had been observed in preliminary experiments that if a small amount of monoethyl hydrogen peroxide was added

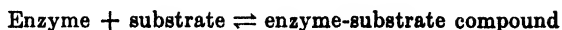
to a highly active catalase solution, the absorption band of the enzyme with the center at  $629\text{ m}\mu$  was not completely extinguished but that a certain residual absorption in this region remained while the absorption bands of the enzyme-substrate compound appeared. The amount of substrate required for complete transformation into the intermediate was determined by adding the substrate solution from a burette to the enzyme solution at low temperature under spectroscopic observation. The titration has to be carried out rapidly, as the breakdown of the intermediate also yields amounts of free enzyme increasing with time.

2.0 cc. of Catalase XXIX ( $k = 7125$ ) required 2.95 and 3.0 cc. of 0.94 N monoethyl hydrogen peroxide (neutralized with phosphate buffer) for the complete temporary abolition of the enzyme absorption band in the red. In a later experiment, 15 cc. of Catalase XXXVI ( $k = 4525$ ) required 6.55 cc. of a 2.07 N peroxide solution. Both titrations were performed at  $3-4^\circ$ .

The number of enzyme molecules present may be computed by using the result of Haldane's (14) calculations, based on Zeile's (4) experimental data, according to which 1 molecule of liver catalase will decompose at  $0^\circ$  and at a substrate concentration of 0.01 M,  $5.4 \times 10^4$  molecules of hydrogen peroxide per second. On Haldane's assumption that 1 enzyme molecule contains 1 iron atom and therefore one active center and with the molecular weight of catalase taken to be 68,000, as found by measuring the rate of diffusion of the enzyme (Stern (15)), it can readily be shown that under the conditions of the assay 1.26 mg. of enzyme will decompose 1 mM of hydrogen peroxide per second. On this basis Catalase XXIX ( $k = 7125$ ) contained 0.214 mg. of enzyme per cc. or  $3 \times 10^{-6}$  mM of catalase. In the titration experiment cited above, 2 cc. of this enzyme solution or  $6 \times 10^{-6}$  mM of catalase required 3 cc. of 1 N monoethyl hydrogen peroxide or 3 mM of the substrate for the optical end-point. Therefore 1 enzyme molecule requires  $5 \times 10^5$  substrate molecules for the complete transformation into the intermediate. From the titration experiment with Catalase XXXVI (see above) a very similar figure may be obtained.

Obviously this ratio does not imply necessarily that 1 molecule of the enzyme-substrate compound consists of 1 catalase molecule and  $5 \times 10^5$  peroxide molecules. It simply means that

an excess of substrate of this order is required to shift the equilibrium in the reaction



entirely to the right. In other words, the probability that each enzyme molecule, whether originally uncombined or whether released on the breakdown of the intermediate, will recombine immediately with fresh substrate molecules, approaches unity. The figure is therefore rather of statistical than of a stoichiometrical character. In particular, it does not indicate how many substrate molecules will combine simultaneously with 1 enzyme molecule.

The figure given may require a correction for two reasons. The lag period mentioned above has not been taken into consideration in the titration experiment. This would tend to make the ratio appear too large. On the other hand, if 1 enzyme molecule does not contain 1 but 4 iron atoms, as does hemoglobin, then the ratio given would have to be multiplied by 4.

*Formation of Intermediate While Enzyme Is Adsorbed*—As has been reported in a recent note (16), the spectrum of the enzyme remains unchanged while it is adsorbed on aluminum hydroxide gel or on silica gel. On addition of monoethyl hydrogen peroxide to the adsorbate in suspension, the peroxide is decomposed and the spectrum of the intermediate is observed as with freely dissolved enzyme. It follows that the combination of the enzyme with the substrate takes place at a grouping of the catalase molecule which is different from that which is attached to the adsorbent. Since the spectrum of the enzyme is not altered by the adsorption but is changed by the addition of substrate, the conclusion appears to be warranted that combination of enzyme and adsorbent takes place by means of the protein carrier of the enzyme, while the substrate combines with the hemin group of the enzyme, causing the specific light absorption in the visible range of the spectrum.

#### DISCUSSION

*Specificity of Catalase*—Catalase has been considered to be the prototype of an enzyme exhibiting an absolute specificity. Heretofore the only substrate known to be attacked by this enzyme was hydrogen peroxide. It has been maintained that neither

alkylated peroxides (17) nor substances of the type of perbenzoic or peracetic acid (18) are affected by catalase. This seemed to provide a significant contrast to peroxidase which in the presence of suitable oxygen acceptors may not only utilize hydrogen peroxide but also ethyl hydrogen peroxide and, to a lesser degree, peracetic acid (19, 20). That monoethyl hydrogen peroxide shows some affinity for catalase was demonstrated several years ago (11). It was then found that the addition of this compound to a system containing catalase and hydrogen peroxide will cause an inhibition of the decomposition of the latter. By varying the hydrogen peroxide concentration alone, it was shown that the inhibition is decreased by increasing the hydrogen peroxide concentration. It was therefore concluded that the inhibitory effect of ethyl hydrogen peroxide is of the competitive type. In those experiments, as in the work of the other authors, highly diluted catalase preparations were used. It was only when the substituted peroxide was added to very concentrated enzyme solutions that the decomposition of this substrate and the formation of a spectroscopically visible intermediate were discovered (3). This fact is proof that the specificity of catalase is of a relative and not of an absolute character. Hydrogen peroxide appears to represent the most readily attacked substrate, while the monoethyl hydrogen peroxide is affected at a much smaller rate. However, as the present study shows, the decomposition of this compound by catalase may be studied in a manner similar to the cleavage of hydrogen peroxide provided sufficiently active enzyme preparations are used.

The physiological function of catalase has hitherto been discussed in a purely speculative manner, inasmuch as all attempts to demonstrate the occurrence of its supposedly unique substrate, hydrogen peroxide, in the cells of higher animals have failed. The demonstration that a substituted organic peroxide may serve as a substrate of this enzyme together with the fact that sufficiently high catalase concentrations to effect such a catalysis have been found to exist in the liver of mammals suggests a new approach to the problem.

*Comparison between Hydrogen Peroxide and Ethyl Hydrogen Peroxide As Substrates of Catalase*—Two points of major interest emerge from the comparative study of the action of liver catalase on these two substrates. Even at low temperatures it has not

yet been possible to observe the appearance of the spectrum of an intermediate when hydrogen peroxide is decomposed. The only explanation suggested at present is a greater rate of breakdown of the enzyme-substrate compound in this case (see Table III).<sup>2</sup> On decomposition of hydrogen peroxide molecular oxygen and water are the reaction products. Monoethyl hydrogen peroxide, on the other hand, yields no gaseous products on enzymatic cleavage but acetaldehyde and other unidentified compounds. The aldehyde formation from a simple organic peroxide is obviously of physiological interest.

TABLE III

*Catalysis of Hydrogen Peroxide and Ethyl Hydrogen Peroxide by Liver Catalase (Over-All Reaction)*

The data for hydrogen peroxide as substrate were taken from the literature (cf. Haldane and Stern (10); Stern (11)).

	Hydrogen peroxide $\text{H}-\text{O}-\text{O}-\text{H}$	Ethyl hydrogen peroxide $\text{C}_2\text{H}_5-\text{O}-\text{O}-\text{H}$
Kinetics of over-all reaction at 0°....	Monomolecular	Monomolecular
“ “ “ “ “ 20°....	“ *	0 order
pH, activity optimum.....	6.5-9	10
1st Michaelis constant ( $K_m$ ), $M$ .....	0.033	0.04
2nd “ “ “ “ “ .....	0.4	Same order
Temperature coefficient, $Q_{10}$ .....	1.4 (0-20°)	2.2 (0-20°)
No. of substrate molecules destroyed by 1 enzyme molecule at 0°, pH 6.6, total substrate concentration 0.02 $N$ .....	$5.4 \times 10^4$	$1.2 \times 10^3 \dagger$

\* The velocity constants are decreasing with time, owing to enzyme inactivation by the substrate.

† Calculated from the determination incorporated in the curve of Fig. 4.

In Table III are compiled some features of the catalysis of both substrates by liver catalase.

*Comparison between Catalase and Methemoglobin As Catalysts—*

<sup>1</sup> In order to observe the spectrum of the intermediate compound the enzyme must be kept saturated with substrate. Since 1 molecule of catalase, if saturated with substrate, splits  $2 \times 10^5$  molecules of  $\text{H}_2\text{O}_2$  per second at 0° (Haldane (14)), for a catalase preparation of  $k = 7000$  a  $\text{H}_2\text{O}_2$  concentration of 6  $M$  would be required for an observation time of 10 seconds. This is experimentally not feasible because the reaction will proceed at an explosive rate and because the enzyme is quickly destroyed by high  $\text{H}_2\text{O}_2$  concentrations.

The methemoglobin catalysis of hydrogen peroxide and ethyl hydrogen peroxide is more than a model reaction. Catalase and methemoglobin have an identical prosthetic group, namely parahematin (12). The much smaller catalytic efficiency of methemoglobin is due to the difference in the nature of the protein carrier. Methemoglobin will only decompose of the order of  $10^{-2}$  hydrogen peroxide molecules per catalyst molecule per second (21). Figures for the ethyl hydrogen peroxide catalysis are not yet available. In contrast to catalase, methemoglobin will form intermediate compounds of a distinct absorption spectrum with both substrates. The pattern of the spectra of the two unstable complexes resembles closely that of the catalase-ethyl hydrogen peroxide compound. The complex of methemoglobin with hydrogen peroxide was discovered 36 years ago by Kobert (22). Its light absorption and composition were recently studied by Haurowitz (23). Haurowitz concludes that the molecular ratio in this case is unity and that the complex contains ferric iron to which the peroxide is linked by coordinative valencies. The complex of methemoglobin with ethyl hydrogen peroxide was recently described by the author (3) and independently by Keilin and Hartree (24). An excess of only 8 peroxide molecules per methemoglobin molecule is sufficient to suppress the absorption band of methemoglobin in the red, compared with an excess of the order of  $10^6$  molecules in the case of catalase. The ratio of these figures resembles that of the catalytic activity of the enzyme and of the blood pigment.

*On Mechanism of Enzyme Reaction*—The present study sheds some light on the mechanism of an enzyme action. The catalyst operates by providing a new path of reaction which leads over an intermediate composed of enzyme and substrate molecules. This compound is unstable but has a mean span of life sufficient to allow for direct observation. It should be emphasized that the interpretation of the observations reported in the present paper is consistent with but not dependent on the validity of the evidence offered for the constitution of the enzyme (4, 12). It is felt that the interpretation is justified by the agreement of the data obtained by the optical and volumetric methods employed. The time required by the spectral cycle to go to completion equals the time required for complete decomposition of the substrate.

The close analogy with the non-enzymatic methemoglobin catalysis suggests a similar constitution of the intermediate compounds. In agreement with the interpretation by Haurowitz (23) of the methemoglobin-hydrogen peroxide complex, the catalase-ethyl hydrogen peroxide complex may be depicted as a covalency compound, where the peroxide molecule is linked to the coordinately tetravalent ferric iron of the hematin group of the enzyme. It appears probable that a similar intermediate occurs during the catalase-hydrogen peroxide catalysis, though hitherto attempts to demonstrate its formation have failed.

It is shown that the rate of formation of the enzyme-substrate compound is rapid compared with the rate of the over-all reaction. The kinetics of the latter are therefore governed by other steps in the series of reactions. The effect of hydrogen ion concentration and of temperature as observed in the study of the total process obviously concerns the later reaction phases. It is quite possible that the actual course of events is more complex than the spectroscopic findings would indicate. The product molecules formed by the breakdown of the intermediate may be radicals, initiating a chain reaction in which the original catalyst no longer participates (11, 25). The dependence of the rate of the over-all reaction on temperature indicates that at any given instant only a fraction of the molecules of the intermediate is in an activated state.

It remains to be seen to which extent the findings of this study apply to enzyme action in general.

#### SUMMARY

1. A study was made of the decomposition of monoethyl hydrogen peroxide by liver catalase. A volumetric procedure was used for the assay of the peroxide. In this manner, the kinetics and the effect of temperature, of pH, of varying the substrate concentration, and of cyanide on the enzyme reaction were studied. The results are compared with those obtained with hydrogen peroxide as substrate.

2. In the course of the enzymatic process there is formed an intermediate compound with a characteristic absorption spectrum. The intermediate is unstable; it breaks down to form free enzyme and reaction products. It exhibits the properties postulated by



Michaelis and Menten for an enzyme-substrate compound. A preliminary optical study of this compound has revealed that it is not a mere adsorption complex; that the rate of formation of the enzyme-substrate compound is great compared with that of the total reaction; that it has a smaller temperature coefficient than the over-all reaction; that it is independent of pH between 4 and 9.

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## A MODIFIED METHOD FOR THE STUDY OF TISSUE OXIDATIONS\*

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There are two common methods for preparing animal tissues for oxygen uptake studies; namely, the "slice technique" and the "mince technique." In the former method the tissues are cut into thin slices about 0.3 mm. thick, care being taken to damage the tissues as little as possible. In the latter method no precautions are taken to avoid damage to the tissues, which are either run through a mechanical mincer (1), cut into fine pieces with scissors (2), or even mashed with a bone spatula (3). Dixon and Elliott (1) stated that the same results were obtained by both the slice and the mince technique, but Elliott and coworkers (4) have since adopted extreme precautions to prevent damage to the tissue, and recommend the slice technique. These workers showed that in a majority of cases the oxygen uptake of minced tissue suspensions was much inferior to that of tissue slices.

The present communication describes a new method for the study of tissue oxidations, which involves (a) the rapid preparation of homogenized tissue suspensions by means of a simple apparatus, and (b) the measurement of the oxygen uptake of the suspension in various dilutions. The new technique for the preparation of the tissue suspension differs from mince techniques in that the tissues are broken down *while in a buffer medium*, and without contamination, inasmuch as the homogenizer is made entirely of glass. It is also possible to have no loss in temperature from the time the animal is killed until the suspensions are in the apparatus.

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† Wisconsin Alumni Research Foundation Fellow.

## EXPERIMENTAL

*The Homogenizer*<sup>1</sup>—This device consists of a motor-driven pestle which fits into an ordinary Pyrex test-tube ( $150 \times 16$  mm.). The pestle is made from a piece of 6 mm. capillary tubing about 220 mm. in length. One end is sealed off and blown into a thick walled cylindrical bulb, whose longitudinal axis is about 20 mm., and which is rounded at the tip to fit the bottom of the test-tube. The sides of the bulb are straight and parallel to the sides of the test-tube for a space of 6 to 7 mm. The bulb can be shaped by placing it in the test-tube while it is still soft and blowing it out to fit the inside dimensions of the test-tube. While the bulb is still hot, about twelve small beads are fused into the tip by applying the tip of a molten glass rod. These small protrusions soon lose their rounded proportions and aid materially in cutting the tissue which is placed in the test-tube. A number of tubes can usually be found to fit the pestle. The fit can sometimes be improved with a little grinding, a fine grade of carborundum and some water being used, and the pestle being driven at a slow speed. The pestle is driven by a cone-drive stirring motor at a speed of 1100 to 1200 R.P.M. The amount of clearance between the pestle and the test-tube in one of our better homogenizers was determined. It was found that the difference between the diameter of the inside of the tube and the diameter of the pestle was 0.23 mm.

To prepare tissue for study, a measured volume of the desired buffer is placed in the homogenizer tube, which is then weighed. The animal whose tissue is to be studied is then killed by decapitation; the desired tissue is quickly excised and a small piece (1 to 2 gm.) is dropped into the buffer in the test-tube. The exact weight of the tissue is then obtained by again weighing the tube. By adding an appropriate amount of buffer, a suspension of any desired concentration can be obtained. In practise, the tissue is partially homogenized in about 4 cc. of buffer, the desired amount of buffer is then run in, and the homogenization is completed.

<sup>1</sup> The homogenizer is a modification of a device used by Dr. Joseph Semb for the preparation of tissues for certain analytical procedures. The authors are also indebted to Dr. L. E. Clifcorn for his suggestions and assistance in the necessary glass-blowing. A similar device for the preparation of fine suspensions of tubercle bacilli has been recently described by Corper and Cohn (5).

During homogenization the test-tube is moved up and down, while the pestle is revolving at high speed. The tissue is torn apart by the protrusions on the end of the pestle and the fragments are kept below the pestle until reduced in size enough so that they can pass by the straight sides of the pestle, where additional grinding takes place. The suspension thus formed settles out very slowly and can be pipetted without difficulty. There are some small bits of connective tissue which tend to clog an ordinary pipette so that pipettes for this purpose are made with slightly enlarged tips. They are also recalibrated for blowout delivery. The apparatus has been used in the study of rat liver and brain, and chick brain, liver, and kidney tissue.

The oxygen uptake was measured in a Barcroft differential respirometer (2). Total volumes of both 3 cc. and 1 cc. were used. The latter volume is to be preferred when rapid uptakes are to be measured.  $\text{CO}_2$  was absorbed by filter paper 3 cm. square, frayed at the end, and placed in the center cups in 0.4 cc. of 10 per cent KOH.

The suspension medium was an  $\text{m}/30$  phosphate buffer at pH 7.4, containing equal moles of sodium and potassium. The contents of the right and left flasks were identical with the exception of the tissue suspension which was placed in the right-hand flask, while a corresponding amount of buffer was placed in the left-hand flask.

*Dilution Effect*—A number of experiments have been carried out with tissue suspensions in concentrations varying from 20 to 200 mg. of tissue per cc. At concentrations over 180 to 200 mg. per cc. the viscosity of the suspension limits the rate of oxygen uptake.

It has been found that when a tissue suspension is diluted, in the absence of added substrate, there is a rapid fall in the oxygen uptake (per gm. of tissue) which is greater than the decrease in tissue concentration (see Fig. 1). This "dilution effect" has also been observed by Krebs (6) in deamination studies on kidney tissue. He observed that in brei which was diluted 4-fold (apparently 125 mg. per cc.) the oxygen uptake was still of the same magnitude as in slices, and that in higher concentrations the uptake sometimes surpassed that of slices. In lower concentrations the uptake decreased rapidly. We have observed the dilution effect in every tissue studied thus far. The relation between oxygen

uptake and tissue concentration is shown in Fig. 1. Representative dilution curves are given for rat liver and brain, and for chick

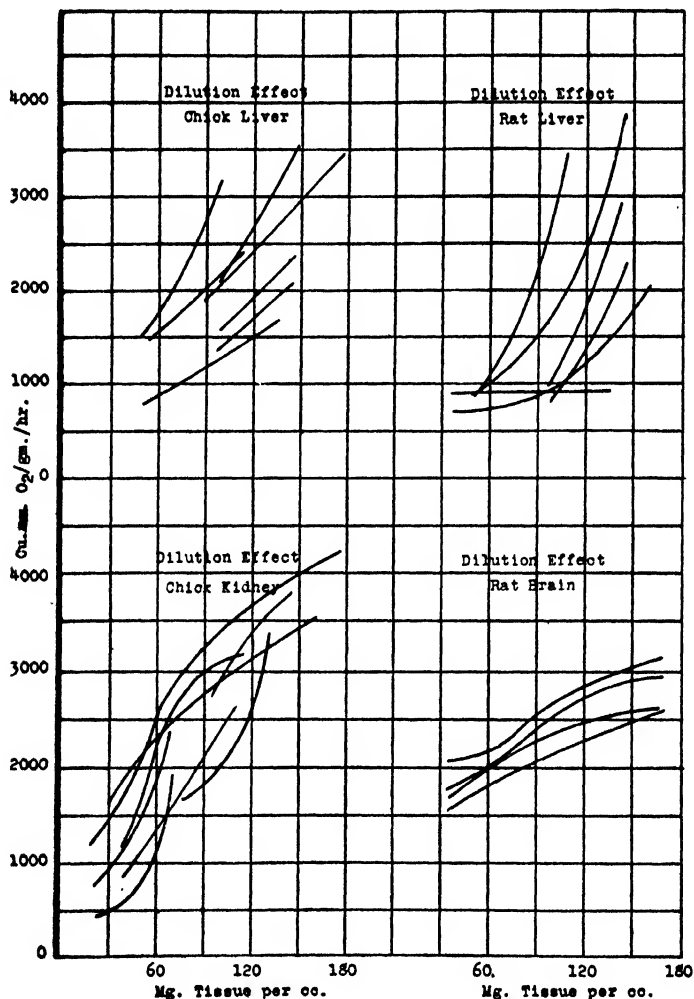


FIG. 1. The dilution effect in chick liver and kidney and rat liver and brain. No added substrate.

liver and kidney. The oxygen uptake is given on the gm. basis. Thus, the dilution curve is a straight line when the uptake is pro-

portional to tissue concentration. The dilution of a tissue suspension cannot be regarded simply as a decrease in the concentration of a one component (*i.e.* tissue) system. If this were true, the oxygen uptake would, of course, be proportional to the amount of tissue present. A suspension of tissue is in reality a complex mixture of systems whose reaction orders are of the first, second, and probably higher order. It seems altogether probable that the dilution effect is a purely physical phenomenon; that is, a natural consequence of the dilution of systems involving two or more reactants. It is only when the reactions of higher order have been eliminated by dilution that one would expect the oxygen uptake to be proportional to the tissue concentration. This appears actually to be the case. When the tissue concentration has been diluted greatly, the only reactions of any consequence which remain are the first order reactions, whose initial rates are proportional to the concentrations of the decomposing compounds. When a suspension has been diluted to the point that oxygen uptake is proportional to tissue concentration, the uptake must represent the oxidations of autoxidizable compounds, and possibly oxidations which do not require activated oxygen and in addition are caused by enzymes which have an extremely high substrate affinity (such as xanthine oxidase).

A reaction of the second order can be reduced to a reaction of the first order by greatly increasing the concentration of one reactant. Likewise, a reaction of the third order can be reduced by increasing the concentration of one or two of the components. In the living cell, "vital phenomena" may be interpreted to some extent as a reduction of the reaction orders by increasing the concentration of various components in the system. In a tissue suspension without added substrate, increasing the tissue concentration causes the oxygen uptake due to binary and ternary reactions to increase exponentially. This increase would presumably continue until each enzyme was in combination with its substrate the greater part of the time, when the decomposition of the enzyme-substrate complex would become a pseudomonomolecular reaction, and become proportional to tissue concentration.

It can be seen from Fig. 1 that there are marked variations in the types of dilution curves for different tissues and there is also considerable variation between samples of the same tissue. There is a definite tendency toward proportionality at low concentrations in

rat liver and chick kidney and there seems to be a tendency toward proportionality in rat brain and chick kidney at the higher concentrations, as discussed above. A number of experiments with rat liver paralleled the experiment in which uptake was proportional to concentration at all levels studied. This may have been caused by a deficient storage of substrate in the tissue.

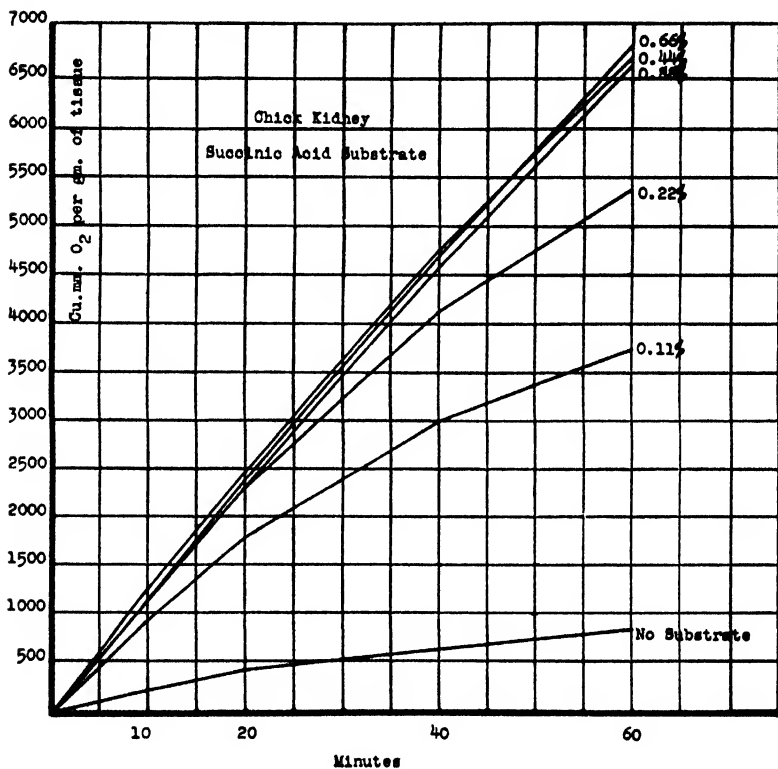


FIG. 2. The oxygen uptake of 44 mg. of chick kidney in 1 cc., with various amounts of succinate substrate.

By diluting the cytoplasmic constituents, the oxygen uptake can be decreased to the point where only the monomolecular reactions are occurring. This uptake is ordinarily quite low. If a particular substrate is now added to the suspension, it should be possible to determine the oxygen uptake due to the reaction between the substrate and the particular enzyme which activates it.

If the enzyme under consideration requires a coenzyme, the mere addition of substrate (without coenzyme) would not be expected to bring about the maximum reaction rate.

*Substrate Experiments*—Since succinoxidase does not appear to require a coenzyme (7), succinic acid was the first substrate to be used with the tissue suspensions. As can be seen in Fig. 1, the oxygen uptake of chick kidney is cut down to a marked degree at concentrations below 50 mg. per cc. Experiments with 0.11 per cent succinic acid showed marked decreases in rate of uptake for successive periods of time. Increasing the amount of substrate to 0.44 per cent gave virtually a constant rate of uptake, as is shown in Fig. 2. The oxidation in the absence of substrate was only 800 c.mm. of  $O_2$  per gm. per hour ( $Q_{O_2} = 4$ ), while the oxidation with succinate substrate was 6800 c.mm. of  $O_2$  per gm. per hour ( $Q_{O_2} = 34$ ). It should be borne in mind that the 800 c.mm. per hour do not represent the true oxygen uptake of the tissue in the absence of substrate, inasmuch as the tissue was at a high dilution. It is quite apparent that the tissue suspension represented a succinoxidase preparation of high activity. At the higher concentrations of substrate there is a tendency for the particles of tissue to clump and for this reason the substrate is generally used at concentrations of 0.3 to 0.6 per cent.

In the presence of an excess of succinic acid the dilution effect could not be demonstrated in any concentrations which were studied (see Fig. 3). The oxidation of succinic acid is apparently proportional to the amount of tissue present, as long as sufficient substrate is present. This is in accord with the concept that the rate of oxidation may be governed by the "decomposition" of one compound (succinoxidase-succinic acid complex). The fact that the oxidation of succinic acid appears to be proportional to the tissue concentration, while the oxidation in the absence of added substrate is not proportional to tissue concentration, leads to the obvious conclusion that substrate differences might be observed at one concentration and not at another. This fact would have considerable bearing on results obtained with the Thunberg technique and is being studied further in that connection.

Elliott and coworkers (4) have reported that minced rabbit kidney showed great diminution in respiration and was almost completely unable to oxidize substrates other than succinate. These



workers did not use various concentrations of tissue and consequently did not observe the dilution effect, although they did

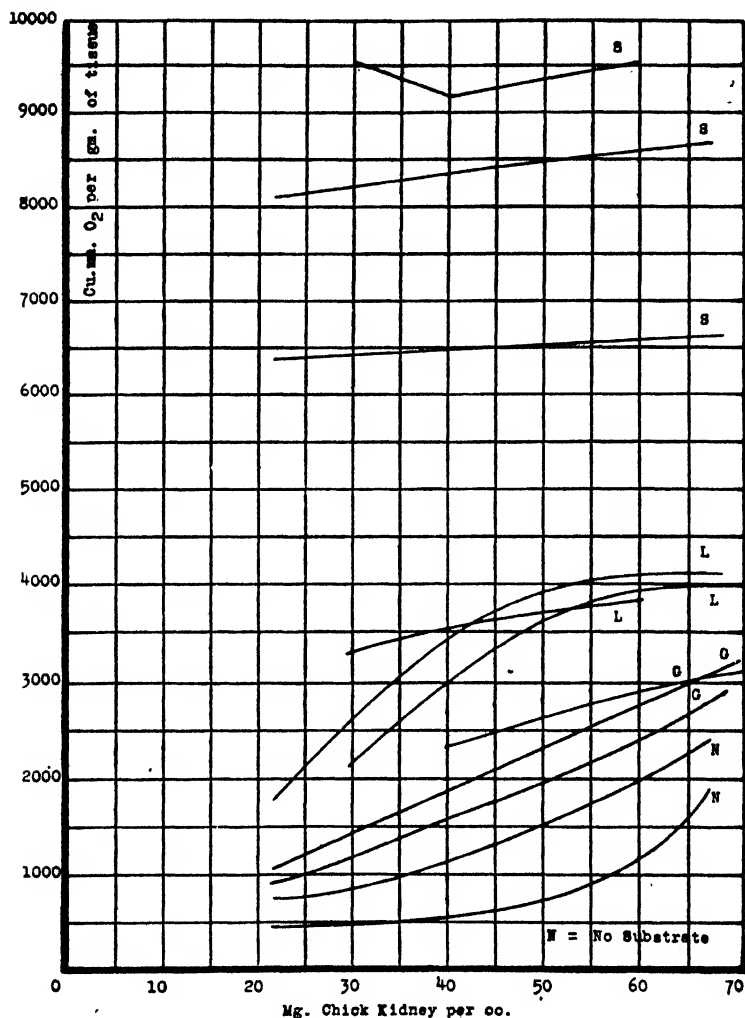


FIG. 3. The oxygen uptake of various amounts of tissue with different substrates. S = succinic acid, L = lactic acid, G = glucose.

remark that the loss of the activity of the enzymes might be due to the dilution of the coenzymes by diffusion. Their results with

minced tissue on no substrate seem unusually low in comparison with their results with slices, inasmuch as their minced tissue was used in fairly high concentration. This may be partly explained by the fact that their tissues were not strictly comparable, since cortex was sliced and whole kidney was minced.

Glucose and lactic acid<sup>2</sup> were used as substrates in a few experiments. The uptake due to these substrates with various tissue concentrations is shown for typical experiments in Fig. 3. Although neither substrate is oxidized as rapidly as succinic acid, the lactic acid appears to be oxidized more rapidly than the glucose. It thus appears that in tissue suspensions glycolysis is the limiting factor in the oxidation of glucose. The coenzymes for glycolysis (adenyl pyrophosphate and  $Mg^{++}$ ) are probably in too high a dilution to be effective under the conditions of these experiments. In view of the fact that lactic acid dehydrogenase requires a coenzyme it is interesting to note that the uptake with lactic acid is as high as it is. The dilution effect was found with both glucose and lactic acid.

It seems entirely possible that the oxidation of glucose and lactate could be brought up to a maximum rate by the addition of appropriate amounts of the required coenzymes. In this way the tissue suspension could be used to study the limiting factors in the reactions which are brought about by the enzyme systems in the tissues, keeping the majority of the other components of the tissues inactive by dilution.

Boyland and Boyland (8) have recently pointed out that results with tissue slices represent at best a balance of conflicting factors. They state that, "The usual method of preparing tissue slices. . . not only produces mechanical injury but also facilitates diffusion from the cells of essential substances such as oxygen carriers, which may thus become diluted beyond their effective or optimum concentration. The smaller the pieces of tissue, the more likely this is. Yet if the tissue slices are not sufficiently thin it is impossible for them to have enough oxygen for their needs when suspended *in vitro*." The experimenter is thus faced with the task of preparing slices thin enough so that oxygen can diffuse *in* freely, while at the same time having the least possible *outward* diffusion of necessary respiratory catalysts, chiefly coenzymes. The new

<sup>2</sup> The *l*(+)-lactic acid was a gift from Dr. E. Tatum, Department of Agricultural Bacteriology, University of Wisconsin.

method of tissue preparation unquestionably permits free access to oxygen and results in a uniform distribution of the coenzymes, which appear to dialyze away from tissues to some extent in *all* methods of tissue preparation. It seems quite probable that previous low results with minced tissue have not been due to destruction of catalytic systems, but rather due to a dilution of the cell contents.

The new method is not proposed as a substitute for the slice technique, which is essentially an attempt to study tissues that are *surviving in vitro* and in which the concentration of the cytoplasmic constituents approaches that found *in vivo*. In surviving tissue slices, however, it is apparent that the reactions observed will be the sum total of a number of reactions all occurring simultaneously. It is felt that at this point the new method can be used to supplement the information given by the slice technique, since it facilitates the study of single enzyme systems, under greatly simplified conditions.

#### SUMMARY

1. A new method for the study of tissue respiration is described in which the tissues are homogenized in a buffer medium by a high speed glass pestle and studied at various dilutions by means of the Barcroft respirometer.

2. The "dilution effect," *i.e.* the lowering of the  $Q_{O_2}$  which occurs when tissue suspensions are diluted, is shown in the case of rat liver and brain, and chick liver and kidney.

3. The applicability of the method to the study of succinoxidase is shown.

4. The possibility of studying other oxidizing systems by adding appropriate coenzymes to tissue suspensions is discussed.

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## THE ANTIRACHITIC EFFECTIVENESS OF VITAMIN D FROM VARIOUS SOURCES\*

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The proper evaluation of antirachitic agents for human use as well as for animals of economic importance is a problem of immediate concern to all interested in rational therapeutics. Chemical assays are as yet out of the question and animal assays reveal a species variation in the degree of reaction to different forms of vitamin D which does not parallel that of the human. Unfortunately as only the vitamin D produced from ergosterol is known in pure form, the difficulties of proper evaluation of an antirachitic agent may be complicated by the presence of unknown forms of vitamin D, the presence of synergistic substances, or the presence of both. It is accordingly important to know how many forms of vitamin D must be recognized, to what extent each occurs in nature or is produced artificially by irradiation, what factors influence the activity of each, and how each may be evaluated properly by laboratory assays with animals.

That irradiated ergosterol was not identical with the vitamin D of cod liver oil was indicated by the work of Massengale and Nussmeier (1) who found that on the same rat unit basis irradiated ergosterol had less antirachitic effect than cod liver oil when tested on chickens. Steenbock and coworkers (2), recognizing the possible synergistic action of other substances in cod liver oil, fed irradiated ergosterol with small amounts of cod liver oil and carotene and obtained no better results than with ergosterol alone. They concluded in consequence that the vitamin D produced by irradiating ergosterol is different from that found in cod liver oil.

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Waddell (3) reported that irradiated cholesterol unit for unit, unlike irradiated ergosterol, was as effective an antirachitic agent for chicks as cod liver oil. This observation has been confirmed (4). He also concluded that the provitamin D of cholesterol was not ergosterol present as a contaminant. Tisdall and collaborators (5) found irradiated cholesterol to be an effective antirachitic for infants. However, Koch and collaborators (6) showed that highly purified non-activatable cholesterol could be converted to a provitamin D which was activatable by ultra-violet radiations. Heating converted "purified" non-activatable cholesterol into a substance activatable with ultra-violet light. These tests were made with rats. Koch and Koch (7) and Hathaway and Lobb (8) recently have also shown that, unit for unit, such cholesterol when irradiated and fed to chicks was more nearly as effective as crude irradiated cholesterol than as irradiated ergosterol. It is not excluded, therefore, that the provitamin D in heated cholesterol is identical with that present in crude cholesterol.

Difficulties in proper evaluation of potency arise even with the naturally occurring vitamins D. Bills and collaborators (9); from studies with chicks, have concluded that the vitamin D activity of fish liver oils may not be restricted to one form of vitamin D. For example, a sample of tuna liver oil was found to be approximately one-sixth to one-eighth as effective with chicks as cod liver oil on an equal unitary basis. Halibut liver oil was found to have an efficiency intermediate between tuna liver oil and cod liver oil.

A great deal of confusion also exists in the assessment of the proper vitamin D efficiency of various vitamin D milks.<sup>1</sup> We (4) have recently reported the relative ineffectiveness of the vitamin D from yeast milk<sup>2</sup> as compared with irradiated milk. These results were obtained with chicks. Similar data for chicks have been presented by Bethke *et al.* (10). However, clinical results have suggested an antirachitic effectiveness for vitamin D milks of a higher order than that shown for cod liver oil even as given with milk (11).

<sup>1</sup> A term used for milk enriched with vitamin D.

<sup>2</sup> Vitamin D milk produced by feeding irradiated yeast to lactating cows.

That the medium in which vitamin D is administered may be a factor in producing different results is indicated by the work of Lewis (12). He reported from a clinical study that calciferol added in propylene glycol to milk was more effective than calciferol in corn oil. More recently Lewis (13) has reported that for the infant crystalline vitamin D incorporated in the daily ration of milk is more effective on a comparable rat unit basis than crystalline vitamin D administered in corn oil or propylene glycol. He has concluded that milk as a medium for the administration of vitamin D increases its effectiveness, and believes that the observed clinical effectiveness of vitamin D milks can be explained on this basis.

The efficiency of synthetic antirachitic agents has also received some attention. Windaus (14) found 22-dihydroergosterol and a 7-dehydrocholesterol prepared in his laboratory activatable by exposure to ultra-violet light. Tested with rats these compounds were approximately one-twentieth to one-thirtieth as active as irradiated ergosterol. Very recently McDonald (15) has reported that rat unit for rat unit irradiated 22-dihydroergosterol had an efficiency for chicks which was intermediate between irradiated ergosterol and cod liver oil. It actually approached the efficiency of cod liver oil more than irradiated ergosterol. Yoder, Thomas, and Lyons (16) have reported the synthesis of a cholesterolene sulfonic acid which is antirachitically active without irradiation with approximately the same degree of effectiveness for the chick as irradiated cholesterol.

#### EXPERIMENTAL

The experiments reported in this paper represent a miscellaneous study including a determination of the antirachitic efficiency of vitamin D from various sources, the effect of certain modifications in the technique of irradiation, an investigation of the alleged beneficial effects of propylene glycol as a carrier for vitamin D, and a correlation of the appearance of certain absorption bands in cholesterol with its activatability.

Parallel assays with rats and chicks were made to compare the antirachitic effectiveness of the various preparations. In our assays with rats we used the curative technique of the 10 day line test. The rats were given Steenbock and Black's (17) basal

rachitogenic Ration 2965. The chick assays were made according to the prophylactic technique with the chick on the basal rachitogenic ration of Hart, Kline, and Keenan (18). The chicks used were white Leghorns, 1 day old, as obtained from commercial hatcheries. They were kept in groups of fifteen in electrically heated brooders with screen bottoms. At the end of 5 weeks their tibias were analyzed for ash after extraction with alcohol.

*Series 1*—In the light of the work of Waddell (3) on cholesterol with chicks, it became of interest to determine the relative effectiveness of irradiated oils of plant and animal origin, since the sterol fractions are credited with containing ergosterol and cholesterol respectively. Of further experimental interest was the fact that while fish oils contain large quantities of cholesterol, yet of these at least one, *viz.* cod liver oil, is well known not to have its vitamin D activity increased by irradiation (19–21).

The following oils or fats were irradiated: soy bean oil, coconut oil, red palm oil, wheat germ oil (hot pressed, cold pressed, and alkali-refined), corn oil, cottonseed oil, peanut oil, sesame oil, lard, chicken fat, cod liver oil, burbot liver oil, halibut liver oil, sardine oil, and tuna liver oil. The chicken fat was rendered and filtered in the laboratory; all the others were obtained from commercial sources. The tuna and halibut liver oils were commercial products sold under the trade names of tuniver oil and haliver oil (Abbott Laboratories) respectively. The former was known to carry a minor admixture of the oils of related species.

The oils were treated as follows: 100 cc. were exposed in a porcelain, flat bottomed dish, with an area of 72 sq. inches, to the radiations from a Hanovia Alpine Sun lamp at a distance of 18 inches for 1 hour. They were stirred every 5 to 10 minutes. Fats solid at room temperature were irradiated at a temperature of approximately 42°. Two of the fish oils, *viz.* commercial tuna liver oil and halibut liver oil, were irradiated in smaller quantities, but the amount of oil per sq. inch was kept constant.

For feeding to our chicks the oils were diluted with Wesson oil so that the requisite dose of vitamin D was incorporated in the rachitogenic diet with the addition of 2 per cent of the diluted oil. The irradiated lard and chicken fat, because of their low activity, were fed undiluted at a somewhat higher level, *viz.* 3.75 per cent. Only four of the irradiated plant oils and one irradiated fish oil,

namely cod liver oil, were included because we felt that sufficient representative data could be obtained from them.

The data obtained with rats are not presented in detail in order to conserve space. In general, however, it may be said that the vitamin D activity of the fish oil failed to be increased by irradiation, while all the others were found to be activatable. It re-

TABLE I

*Series 1. Ash in Tibia of Chicks Fed Vitamin D from Irradiated Plant and " Animal Oils*

Additions to basal ration	International units per 100 gm ration*	No. of chicks	Average weight of chicks	Average weight of ash	Average ash per cent
			gm.	gm.	
None . . . . .	0	14	170	0.1534	30.58
Cod liver oil . . . . .	8 25	14	240	0.2808	38.35
Irradiated cod liver oil . . . . .	8 25	15	219	0.2551	37.44
Cod liver oil . . . . .	16.5	15	275	0.3785	46.40
Sardine liver oil . . . . .	16.5	15	241	0.2950	43.27
Burbot " " . . . . .	16 5	15	250	0.3335	44.69
Halibut " " . . . . .	16 5	14	218	0.2514	42.20
Tuna " " . . . . .	16 5	13	228	0.2789	40.34
Irradiated chicken fat . . . . .	16.5	15	258	0.3328	44.37
" lard . . . . .	16 5	13	218	0.2436	38.82
" coconut oil . . . . .	82 5	14	174	0.1681	34.71
" wheat germ oil . . . . .	82 5	14	212	0.2157	35.20
" peanut oil . . . . .	82.5	14	186	0.1867	34.57

The difference in effectiveness between irradiated chicken fat and irradiated lard was explained by our final rat assays wherein it was found that our preliminary assays were in error. It was found that the irradiated chicken fat was approximately twice as effective as we had believed.

\* Originally determined by assay with rats as Steenbock units and converted to international units by the factor 3.3.

quired from 15 mg. (coconut oil) to 750 mg. (soy bean oil and lard) to give an antirachitic activity equivalent to 1 Steenbock unit (3.3 international units) of vitamin D.

The data obtained with chicks are presented in Table I. They show, first, that the vitamin D activity of cod liver oil for the chick was not increased by irradiation; secondly, that the effectiveness of the vitamin D in various fish oils for chicks was approximately



the same as for rats, unit for unit. Commercial tuna liver oil and pure halibut liver oil, unit for unit, were somewhat less effective than the other oils. The differences were, however, not so great as those reported by Bills, Massengale, and Imboden (9). They investigated the liver oil from the blue fin tuna and reported a material difference in the unit for unit effectiveness between it and cod liver oil. Very recently Bills (22) has reported that for the chick the antirachitic effectiveness of tuna liver oils was determined by species. That from one species, the blue fin tuna, was approximately one-eighth as effective as cod liver oil, whereas that from other species was found to be more effective than cod liver oil on an equivalent rat unit basis. Whether species differences account for the discrepancy between our results and the early results of Bills and collaborators we, unfortunately, could not determine. Thirdly, our results show that the provitamin D of animal fats is different from that in plant oils, the former resembling cholesterol and the latter ergosterol with respect to their antirachitic effect on chicks. Bethke *et al.* (23) in a recent paper have reported similar results.

*Series 2*—That the solvent in which ergosterol is dissolved influences the rate and efficiency of vitamin D synthesis with exposure to ultra-violet light was reported by Bills *et al.* (24) in 1931. He presented time-activation curves for ethyl alcohol, ethyl ether, and cyclohexane as determined with rats by the line test. We questioned whether the products produced were identical forms of vitamin D. We consequently irradiated cholesterol and ergosterol in ethyl ether and ethyl alcohol solution (0.1 per cent) and in addition in crystalline form. The irradiation was carried out with a Hanovia Alpine Sun lamp at a distance of 18 inches for 1 hour. The crystals were stirred every 5 to 10 minutes during the irradiation period. The resultant products were fed to chicks on an equal unitary basis, as determined with rats. Table II shows that the solvents had no effect upon the character of the vitamin D produced.

We have recently reported that skim milk fed with various sources of vitamin D had no effect on their comparative antirachitic effectiveness for chicks. Lewis (12), however, has reported that calciferol added to milk in propylene glycol solution was approximately twice as effective for the infant as calciferol ad-

ministered in corn oil solution. We anticipated that propylene glycol might have an effect in the absence of milk. We accordingly fed calciferol and irradiated cholesterol both in propylene glycol and Wesson oil solutions. The propylene glycol solutions were incorporated into the basal chick rations to the extent of 1

TABLE II

*Series 2. Ash in Tibia of Chicks Fed Vitamin D from Cod Liver Oil, Irradiated Cholesterol, Irradiated Ergosterol, and Calciferol*

Additions to basal ration	International units per 100 gm. ration	No of chicks	Average weight of chicks	Average weight of ash	Average ash
			gm.	gm.	per cent
None . . . . .	0	12	168	0.1738	31.12
Cod liver oil diluted with Wesson oil . . . . .	16.5	14	265	0.3609	43.64
"      "      "      "      propylene glycol . . . . .	16.5	15	279	0.3764	44.33
Cholesterol irradiated as crystals and fed in Wesson oil solution . . . . .	16.5	15	255	0.3593	45.88
Cholesterol irradiated as crystals and fed in propylene glycol solution . . . . .	16.5	15	245	0.3301	43.88
Cholesterol irradiated in alcohol and fed in Wesson oil solution . . . . .	16.5	15	265	0.3574	45.12
Heated purified cholesterol irradiated as crystals and fed in Wesson oil solution . . . . .	16.5	15	257	0.3617	45.24
Ergosterol irradiated in alcohol and fed in Wesson oil solution . . . . .	165	15	224	0.2538	37.67
Ergosterol irradiated as crystals and fed in Wesson oil solution . . . . .	165	14	230	0.2655	38.32
Ergosterol irradiated in ether and fed in Wesson oil solution . . . . .	165	15	217	0.2498	39.08
Calciferol fed in propylene glycol solution . . . . .	165	15	210	0.2260	36.57
Calciferol fed in Wesson oil solution . . . . .	165	13	232	0.2761	39.30

per cent of the diet in the same manner as our oil solutions. Cod liver oil emulsified with propylene glycol instead of the usual Wesson oil was also included in this series. Table II shows that propylene glycol was no more efficient carrier for vitamin D than Wesson oil.

Koch and his collaborators (6, 25) have reported that the pro-

vitamin D content of cholesterol purified by bromination could be increased by heating it above its melting point. Their data on absorption spectra revealed that no spectral absorption characteristic of ergosterol, in fact, no banded absorption, was produced in the purified and heated preparation. We repeated Koch's experiments using chicks as well as rats to determine

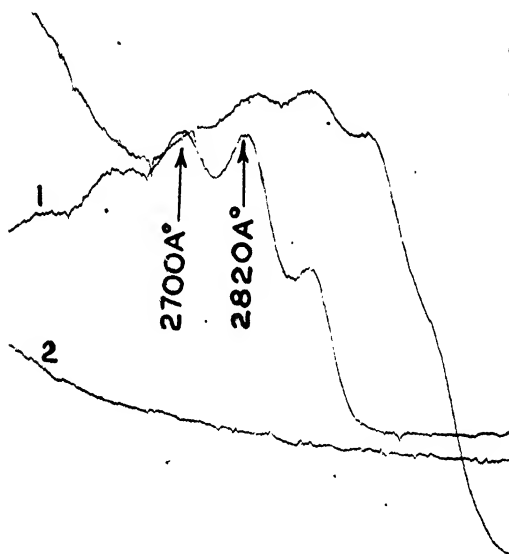


CHART 1. Absorption spectra as reproduced from the original photographs with a Moll microphotometer. Curve 1 represents 2 per cent crude cholesterol, 10 mm. thickness; Curve 2, 2 per cent purified cholesterol, 10 mm. thickness; Curve 3, 0.5 per cent purified, heated cholesterol, 10 mm. thickness.

whether the vitamin D produced from heated purified cholesterol was identical with that produced from crude cholesterol. Although Koch *et al.* (6, 25) failed to find absorption bands in his heated purified cholesterol, we (Chart 1) found four bands spaced similarly to those of crude cholesterol but shifted somewhat to the red end of the spectrum. The possibility was not excluded, therefore, that irradiation of these cholesterol might have produced a different vitamin D. A purified cholesterol therefore was

prepared according to the bromination technique of Bills *et al.* (26). After irradiation this was found to produce no response with the line test, when fed at a 50 mg. level. However, after we had heated and irradiated the preparation according to Koch's (6, 25) technique, 1 mg. of the irradiated preparation was found to have a potency of 3.3 international units. This preparation when fed to chicks (Table II) produced calcification more closely resembling in amount that induced by irradiated crude cholesterol than ergosterol. These results confirmed those of Hathaway and Lobb (8) which appeared after our experiments were completed.

#### SUMMARY

1. The antirachitic activity of certain fish oils, *viz.* cod liver oil, halibut liver oil, tuna liver oil, burbot oil, and sardine oil, was not increased by irradiation.

2. The comparative antirachitic effectiveness of the fish oils, as determined with chicks and rats, was approximately the same. However, the commercial tuna liver oil that we fed was somewhat less effective than the other oils studied.

3. Irradiated plant oils, *viz.* coconut oil, wheat germ oil, and peanut oil, were less effective antirachitically for chicks than irradiated animal fats, *viz.* lard and chicken fat, on an equivalent unitary basis.

4. Propylene glycol as a solvent for vitamin D did not increase its effectiveness.

5. The solvent, in which ergosterol or cholesterol was dissolved during irradiation, had no influence upon the relative antirachitic effectiveness of the resultant vitamins D as determined with chicks.

6. Purified cholesterol, heated and then irradiated, produced a response in chicks more like that produced by irradiated crude cholesterol than irradiated ergosterol.

7. Absorption spectra are presented which show the presence of absorption bands in heated purified cholesterol.

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## SOME APPLICATIONS OF A NEW COLOR REACTION FOR CREATININE

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Some years ago it was found by one of us that creatinine reacts with 3,5-dinitrobenzoic acid in alkaline solution to give a purplish rose color, and that the reaction is sufficiently sensitive to permit its application to urine and blood filtrates.<sup>1</sup> Particular interest is attached to a new reaction for creatinine because the Jaffe reaction with picric acid has hitherto been the only known highly sensitive reaction for this compound, and because the determination of creatinine is practically wholly dependent upon the use of this single reaction which, as is well recognized, is not highly specific for creatinine. The present paper describes results obtained by the application of the dinitrobenzoic acid reaction to creatinine and to various compounds closely related to it, as well as to urine and blood filtrates.

All of the creatinine derivatives and related substances which were studied reacted either positively or negatively to both picric and dinitrobenzoic acids, which indicates a similarity in the mechanism of the two reactions. However, the reaction with dinitrobenzoic acid appears to be somewhat more specific for creatinine, as evidenced by our results with urine and blood filtrates, described below, and by the fact that all of the reactive creatinine derivatives which were studied gave somewhat less color, in terms of creatinine, with the new reagent than with picric acid.

<sup>1</sup> The finding that creatinine reacts with 3,5-dinitrobenzoic acid in presence of alkali is contrary to the result reported in this connection by Greenwald and Gross (1). In a private communication to the authors, Dr. Greenwald has stated that his negative results with 3,5-dinitrobenzoic acid were apparently due to his having used an incorrectly labeled sample of material when his test was made.

Contrary to its behavior with picric acid, glucose, even in high concentrations, fails to give color with dinitrobenzoic acid either in the cold or upon heating. Acetone and diacetic acid react positively to the new reagent. These substances also have an effect upon the picric acid reaction. The reaction with 3,5-dinitrobenzoic acid is more affected by the presence of other substances, such as neutral salts, than is the reaction with picric acid. Like picric acid, 3,5-dinitrobenzoic acid is reduced by strong reducing agents such as metallic zinc and hydrogen sulfide.

In contrast to the picrate reaction with creatinine, the new reaction is characterized by a more or less rapid fading of color after maximum development. The colored product is photo-sensitive. In absence of direct sunlight, however, the rapidity of both the increase and the fading of color is largely determined by the concentration of alkali present. By using suitable amounts of the reagents, creatinine can be detected in a concentration of 0.01 mg. per cent. Sufficient color for colorimetric readings is developed in creatinine concentrations of about 0.2 mg. per cent or slightly less. Under the conditions employed for these determinations, practically no blank color is developed by the reagents.

For the determination of urinary creatinine, the new reaction has the advantages over picrate methods of a greater specificity and a more easily readable color. The instability of the color toward strong light and its sensitivity to variations in method, however, impose a more rigorous technique than is necessary in picrate methods and make it doubtful whether the new method, at least in its present form, will be favored as a routine substitute for the picrate methods. As a possible alternative method and as a check upon the validity of results by the picrate method, as well as in studies involving the differentiation of creatinine from closely related compounds, the new reaction should prove to be of value.

#### *The Reaction Applied to Various Compounds Related to Creatinine*

We have studied the reaction of the following creatinine derivatives<sup>2</sup> with both the picric acid and dinitrobenzoic acid methods.

The tests were carried out on relatively dilute solutions of these

<sup>2</sup> In this connection we wish to express our indebtedness to Dr. I. Greenwald who generously supplied us with samples of these compounds.

compounds by (a) the original Folin method for blood (2) in which alkali is added to a saturated picric acid solution containing the material tested, (b) the Folin-Wu blood method (3) in which 5 cc. of an alkaline picrate solution are added to 10 cc. of filtrate or water solution, and (c) the dinitrobenzoic acid method carried out according to the technique described later in connection with its application to blood.

The results with the creatinine derivatives were as follows:

*Benzal creatinine*, *acetylbenzal creatinine*, *tribenzoylcreatinine*, and *creatinine oxime* fail to react with either reagent.

*Methyl-*, *dimethyl-*, and *ethylcreatinine hydroiodide*, 4- (or 5-) *benzoylcreatinine*, 5-*benzylcreatinine hydrochloride*, and 2-*benzylcreatinine* give positive reactions with both reagents. Greenwald (4) reported a positive or negative reaction with picric acid for some of these compounds, and our results corroborate and extend those observations.

For the reactive derivatives, except in the case of 2-benzylcreatinine, the rates of color development with both reagents are markedly different from that of creatinine, but show a striking resemblance to each other. The creatinine derivatives, with the above exception, react within the first minute with both reagents to give an intense initial color which fades as compared with simultaneously prepared creatinine solutions during the following 9 minutes. In the picrate reaction this appears to be a slowing up or cessation of color development and after 10 minutes the readings tend to be constant against creatinine standards. In the dinitrobenzoic acid reaction the fading is in some cases an actual as well as a relative one, accompanied by a change in type of color. The colors from the derivatives did not exactly match that of creatinine by either method and differed with the different compounds, but the differences were in most cases greater with the dinitrobenzoic acid method than with the picric acid method. As a result, the readings against creatinine standards are difficult and in the case of the dinitrobenzoic acid method become almost impossible after 15 minutes.

2-Benzylcreatinine was exceptional among the creatinine derivatives studied in that its rate of color development in both the picric acid and dinitrobenzoic acid reactions was very similar to that of creatinine. Colorimetric readings against simultaneously pre-



TABLE I  
*Approximate Creatinine Equivalents of Creatinine Derivatives and Related Compounds during Development of Color in Picrate and Dinitrobenzoate Reactions. Determined by Readings against Simultaneously Prepared Creatinine Standards*

The figures represent mg. of creatinine equivalent to 1 mg. of the compound.

Reacting compound	With picrate (Folin-Wu blood method)					With dinitrobenzoate				
	1	5	10	15	20	1	5	10	15	20
After addition of alkali, min. . . . .										
Methylcreatinine Hl. . . . .	1.8	0.36	0.35	0.33	0.32	1.1	0.35	0.15	0.12	0.08
Dimethylcreatinine Hl. . . . .	0.66	0.43	0.34	0.32	0.29	1.0	0.2	0.11	0.08	0.05
Ethylcreatinine Hl. . . . .	1.17	0.45	0.4	0.4	0.42	1.1	0.46	0.26	Unreadable	
4- (or 5-) benzoylcreatinine* . . . . .	0.87	0.53	0.5	0.5	0.5	0.85	0.36	0.23	0.15	Unreadable
5-Benzylcreatinine HCl . . . . .	1.3	0.55	0.48	0.4	0.4	1.5	0.5	0.13	0.11	0.1
2-Benzylcreatinine† . . . . .	0.12	0.1	0.1	0.1	0.1	0.02	0.01	0.01	0.01	0.01
Glycocylamide HCl. . . . .	0.2+	0.4	0.51	0.58	0.6	0.14	0.12	0.1	0.08	0.07
Hydantoin. . . . .	0.004	0.007	0.01	0.018	0.02	0.005	0.006	0.007	0.009	

\* Insoluble in water; dissolved in about 1 per cent alcohol.

† Insoluble in water; dissolved in about 0.002 N HCl.

pared creatinine standards were constant for at least 20 minutes, except for a slightly more rapid increase of color in the 2-benzyl-creatinine during the first 5 minutes. With picric acid the color agreed well with the standards and the readings were constant for at least 45 minutes. With dinitrobenzoic acid the color match was not so close and the difference in shade became greater after 20 minutes. Approximate creatinine equivalents for various creatinine derivatives are shown in Table I.

Curves for the rate of color development of creatinine and of 2-benzylcreatinine were constructed on the basis of readings obtained with the photoelectric colorimeter of Goudsmit and Summerson (5). By the Folin-Wu blood creatinine method the curves of these two compounds are strikingly similar, as is shown in Fig. 1. Our work here convinced us, contrary to previously reported findings, that the development of color from creatinine and picric acid is not fully complete until 12 minutes after the addition of the alkali, although the increase between 10 and 12 minutes is very slight. It is possible that the photoelectric colorimeter has brought out this slight increase in color which has escaped notice with the visual instruments. After 7 minutes the two curves are practically identical and continue so for at least 20 minutes. The readings for different determinations made in the construction of these curves could be duplicated to within 1 to 2 per cent. With dinitrobenzoic acid the similarity of the curves is less marked.

In addition to these creatinine derivatives, samples of glyco-cyamidine hydrochloride, glyco-cyamidine, and hydantoin<sup>3</sup> were tested in the same way with the following results.

Glyco-cyamidine hydrochloride reacted with both picric acid and dinitrobenzoic acid, giving more color (in terms of creatinine) with the former. By both picrate methods the color increased more rapidly than that of creatinine during the first 15 minutes, after which the increase is apparently very slight but noticeable. The shade of color was almost, but not quite, similar to that given

<sup>3</sup> The glyco-cyamidine which we used was a commercial product prepared by Schuchardt. Our thanks are due to our colleague, Dr. Richard W. Jackson, for preparing the specimens of glyco-cyamidine hydrochloride and hydantoin employed in this study. The glyco-cyamidine hydrochloride was prepared essentially according to Schmidt (6) and gave the following analysis: Cl, calculated 26.16; found 26.22. The hydantoin melted at 221-222°.

by creatinine. With dinitrobenzoic acid the glycoyamidine color fades with respect to creatinine standards and is much browner, the match becoming poorer as time goes on, so that all the readings recorded are only approximate. Creatinine equivalents of glycoyamidine hydrochloride by the Folin-Wu and dinitrobenzoic acid methods are shown in Table I.

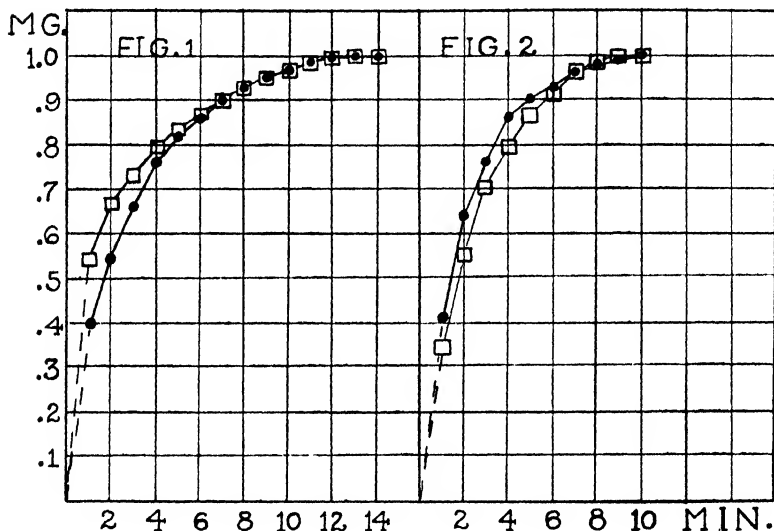


FIG. 1. Curves for rates of color development of creatinine and of 2-benzylcreatinine with the Folin-Wu blood picrate method, from figures obtained by use of a photoelectric colorimeter. ● represents creatinine; □ represents 2-benzylcreatinine.

FIG. 2. Curves reproduced from Hayman, Johnston, and Bender's figures (8) for rates of color development of creatinine and of the serum chromogenic material extracted from Lloyd's reagent (figures for the latter have been recalculated on the basis of a maximum color development of 1 mg.). ● represents creatinine; □ represents material extracted from Lloyd's reagent.

*Glycoyamine* gave no color by either method, but in a single determination made after evaporation to dryness with HCl, as in the creatine determination (7), results similar in character to those of glycoyamidine were obtained. The results here indicated that only 10 per cent of the glycoyamine had been converted into glycoyamidine.

*Hydantoin* in sufficiently concentrated solutions reacts with both reagents, giving relatively more color per mg. with picric acid than with dinitrobenzoic acid. The color with both reagents increases steadily for long periods of time. The type of color with alkaline picrate is similar to that of creatinine. With dinitrobenzoic acid it is similar for 5 or 6 minutes, after which the match becomes difficult. Creatinine equivalents are shown in Table I.

The comparative curves for rate of color development by creatinine and 2-benzylcreatinine (Fig. 1) seem to us to bring out at least one point of real interest. This is that a close similarity in the rates of reaction of creatinine and of an unknown compound affords little real evidence that the unknown reacting compound is creatinine. Thus, it may be noted that the curves of creatinine and of 2-benzylcreatinine obtained by the picrate method approximate each other about as closely as do the curves for creatinine and for the material released from Lloyd's reagent as reported by Hayman, Johnston, and Bender (8).<sup>4</sup> (See Fig. 2.) A further point of interest in connection with our work with 2-benzylcreatinine and with glyocyamidine is the greater specificity for creatinine of the dinitrobenzoic acid method, both in rate of reaction and in the shade of color produced, as compared with results by the picrate method. These findings are of especial interest in connection with the fact, reported later in this paper, that blood filtrates fail to yield a typical creatinine color reaction when tested by the dinitrobenzoic acid method.

#### *Method for Determination of Urinary Creatinine and Creatine*

We have used the following technique for the determination of creatinine in urine by the dinitrobenzoic acid method. In this connection it may be pointed out that the conditions adopted for the determination as applied to urine do not provide for development of the maximum amount of color from the creatinine present. The color produced is, however, strictly proportional to the amount of creatinine present. To provide for maximal color production from the creatinine would require a very high dilution of the urine,

<sup>4</sup> In order to facilitate comparison with their creatinine curve, the figures given by Hayman, Johnston, and Bender for material released from Lloyd's reagent were recalculated on the basis of a color equivalent to 1 mg. at 10 minutes.

and we believe that the technique which we have adopted is preferable to one involving such dilution.

*Reagents—*

1. A stock creatinine solution containing 0.1 per cent creatinine in 0.1 N HCl. From this a 25 mg. per cent standard (2 cc. of which contain 0.5 mg. of creatinine) is prepared daily by a dilution of the stock solution with water.

2. A 1 per cent solution of purified 3,5-dinitrobenzoic acid<sup>5</sup> in 95 per cent alcohol, kept in a brown glass-stoppered bottle.

3. A 6 per cent solution of NaOH.

*Determination*—The urine is diluted so that approximately 0.5 mg. of creatinine is contained in 1 to 2 cc. Normal 24 hour samples usually require a dilution of 1:1. Samples with a high specific gravity should usually be diluted 1:4. If the specific gravity is less than 1.010, dilution is usually unnecessary. 2 cc. of the dilute creatinine standard (containing 0.5 mg. of creatinine) are transferred to a small, dry flask and 1 and 2 cc. portions of the diluted urine measured into two similar flasks. 1 cc. of water is added to the flask containing 1 cc. of urine, and 3 cc. of the dinitrobenzoic acid reagent are added to each of the three flasks. 1 cc. of 6 per cent NaOH is then added from a burette or pipette to each

<sup>5</sup> The most satisfactory samples of 3,5-dinitrobenzoic acid which we have been able to purchase were the c.p. product of the Eastman Kodak Company. This product does, however, contain material which yields a blank with alkali and before use should be purified by recrystallization from glacial acetic acid as follows: A 30 per cent solution of the product in glacial acetic acid is made with the aid of heat, stoppered with cork, and allowed to stand, without shaking, at room temperature for at least 12 hours. The crystals are collected on a Buchner funnel on hard filter paper, sucked dry, and washed with several portions of ice-cold distilled water until there is no longer any odor of acetic acid. They are then sucked as dry as possible, transferred to a watch crystal, and dried in an oven at about 90° for 30 to 45 minutes. The pure product should be preserved in a brown glass-stoppered bottle. The yield of crystals after 12 hours standing in glacial acetic acid is about 55 per cent of the original material; after 48 hours, about 58 per cent; and after 8 days, about 67 per cent. After this no further crystallization occurs but the flask may be allowed to stand undisturbed for at least 10 days. All of the product obtained in this way is suitable for use in this method. An additional yield can be obtained by the addition of water to the supernatant liquids and washings. The fine crystals which settle out from these may be collected and recrystallized from glacial acetic acid as described above, to give a satisfactory product.

flask at as nearly the same time as is possible and the contents of the flasks mixed by rotation. The solutions should not be exposed to direct sunlight during the reaction or before the reading is made. At the end of from 10 to 12 minutes, 10 cc. of distilled water are added to each flask by means of a freely flowing burette. The time elapsing between the dilution of the first and last flasks should not exceed 45 seconds. The contents of the flasks are mixed by inversion and a colorimetric reading made within 8 minutes after dilution. The concentration of the unknown solution should be within 50 per cent of that of the standard. Creatinine solutions of twice and one-half the value of standards containing either 0.5 mg. or 1.0 mg. of creatinine read to within 95 to 98 per cent of the correct values, by this method. If test-tubes graduated to 15 cc. are available, the determination may be carried out in these, with final dilution to volume.

Creatine and glucose even in excessive amounts give no color in this method. Acetone and diacetic acid react to some extent with the reagent and should be removed if present in large amounts. None of the diabetic urines which we have encountered contained enough of these substances to cause interference.

Table II shows representative creatinine figures by the new method as compared with the two commonly used picrate methods. These figures are typical of those obtained in the analyses of 63 normal and pathological urine samples. Picrate Method I is the original Folin method (9), except that the volumes were reduced to one-fifth of those called for in the original directions and creatinine standards were used instead of dichromate. Picrate Method II is Folin's modification of his original method (10). In this modification, the amounts of picric acid and sodium hydroxide are increased over those used in the original method, about 7 times as much saturated picric acid and 1.5 times as much alkali being present during the reaction. The three methods were carried out on the same sample of diluted or undiluted urine, the same standard solutions being used. The diabetic urine samples contained from 0.5 to 3 per cent sugar and considerable amounts of acetone and diacetic acid.

In attempting to test the accuracy of the new method and of the picrate methods for creatinine as applied to urine, we have adopted a procedure, which has not, we believe, been hitherto applied to

TABLE II

*Representative and Average Figures for Creatinine in Normal and Pathological Urine and for Recovery of Added Creatinine by the New Method and by Two Picrate Methods*

The figures represent mg. of creatinine per 100 cc. of urine unless otherwise stated. All of these determinations were made on human urine except Specimen 8.

Specimen No.		Urinary creatinine			Creatinine added to	Creatinine added	Recovery of added creatinine		
		Picrate Method I	Picrate Method II	New method			Picrate Method I	Picrate Method II	New method
1	Normal	75.5	79.0	72.2	Lloyd's filtrate	100	102.6	105.8	100.2
2	"	112.4	113.6	109.3	" "	100	101.5	105.8	99.7
3	"	123.0	124.1	117.5	" "	130	133.2	133.2	131.6
4	"	123.5	129.0	117.9	Urine	125	125.4	122.9	123.5
5	"	141.1	142.3	137.4	"	100	101.2	99.6	101.8
6	"	199.0	199.5	184.4	Lloyd's filtrate	100	103.6	107.5	101.5
7	"	239.4	248.5	238.6	" "	200	207.2	207.2	201.8
8	" dog	180.6	185.2	173.5	Urine	200	202.0	204.0	202.8
9	Nephritis	58.2	59.5	55.5	Lloyd's filtrate	200	205.2	212.2	204.0
10	"	128.2	130.7	122.7	Urine	200		188.0	205.6
11	Tuberculosis of kidney	100.7	105.3	96.8	Lloyd's filtrate	100	101.0	103.3	100.0
12	Urological (?)	54.5	55.4	52.5	" "	50	50.0	51.1	50.4
13	Gallbladder disease	258.0	258.0	242.4	" "	100	105.8	107.2	100.0
14	Appendectomy	227.8	237.2	222.2	" "	100	102.0	103.9	100.5
15	Myelogenous leukemia	86.3	99.1	80.7	" "	100	103.1	102.1	100.7
16	Thyroidectomy	171.4	171.9	162.2	" "	200	212.0	216.2	200.4
17	Muscular dystrophy*	22.4	23.0	19.7	" "	100	197.4	199.0	194.0
18	Diabetes	49.5	59.3	51.7	" "	100	105.0	107.2	102.2
19	"	90.5	92.3	82.0	Urine	175	173.8	181.8	177.0
20	"	103.1	111.1	100.0	" "	25	26.6	26.3	25.1
21	"	113.6	118.0	108.7	Lloyd's filtrate	25			25.0
22	"	127.6	130.9	121.5	" "	100	102.0	101.5	100.1
					" "	100	99.2	106.1	99.3
					" "	100	103.9	108.1	100.0
					" "	100	102.8	106.1	99.6
					" "	100	102.8	105.3	99.0

\* A 24 hour sample contained 835 mg. of creatine.

urine. This procedure involved extraction of the urine with Lloyd's reagent under conditions which assure removal of all creatinine, and then determination of the creatinine content of the urine filtrates both before and after addition of known amounts of creatinine.

1 volume of urine was diluted with an equal volume of 0.05 N oxalic acid, and with additional water if necessary to make the same dilution as that used in the original creatinine determination. This was shaken for 4 or 5 minutes with Lloyd's reagent (15 gm. per 100 cc. of solution) and the mixture was then filtered and this process repeated. 20 per cent sodium carbonate was added to the final filtrate drop by drop until no further precipitate formed, and the mixture was then filtered. The filtrate obtained here was made neutral or slightly acid to litmus with HCl and a portion was accurately measured into a flask, an equal volume of distilled water being measured into another flask. Small, identical amounts of a concentrated solution of creatinine were added from the same pipette to both flasks. Typical figures for the recovery of creatinine added directly to the urine and to filtrates from urine after the treatment with Lloyd's reagent are included in Table II.

Figures for urinary creatinine were, with few exceptions, slightly lower by the new method than by either of the picrate methods. The average figure of 63 determinations by the original picrate method was 125.1, by the modified picrate method 128.3, and by the new method 121.8 mg. per 100 cc. of urine. The highest figures were almost always given by Folin's modified picrate method. Recoveries of creatinine, whether added to urine or to urine filtrates treated with Lloyd's reagent, averaged (for twenty-seven determinations) 103.5 per cent by the picrate methods and 99 per cent by the new method. Filtrates treated with Lloyd's reagent, to which no creatinine was added, were, by the new method, barely distinguishable from a water blank with the same reagents, being in no case comparable with standards corresponding to as much as 2 mg. of creatinine per 100 cc. of original urine. With the picrate methods the same filtrates showed color equivalent to from 4 to 9 mg. of creatinine per 100 cc. of original urine and figures for creatinine added to these filtrates are correspondingly higher than the creatinine standards. The amount of this picrate-reacting material not removed by Lloyd's reagent is



enough to account for the difference between picrate and dinitrobenzoate figures on the original urine. The picrate-reacting substance is evidently neither creatinine nor a product from the Lloyd's reagent, since filtrates from pure solutions of creatinine which have been treated with Lloyd's reagent in the same way give no more color by picrate methods than water similarly treated. The picrate-reacting substance is not glucose, since amounts of this compound corresponding to 2 per cent in the original urine do not increase creatinine color in either of the picrate methods.

Creatine added to normal urine and the creatine excreted by a hospital patient with myxedema<sup>6</sup> were determined by the three methods as described above after conversion of creatine to creatinine by evaporation to dryness with HCl and lead, according to the method of Benedict (7). The determination of total creatinine by the new method was carried out exactly as was that of preformed creatinine. Rochelle salt should not be added to the alkali, since in the high concentration used for the picrate methods it affects the reaction, whereas traces of lead do not. The results obtained with the creatine-containing urines are shown in Table III.

#### *Application of the 3,5-Dinitrobenzoic Acid Reaction to Blood Filtrates*

The dinitrobenzoic acid reaction has also been applied to blood filtrates. The procedure adopted for this work was as follows: To 5 cc. of a 1:5 tungstomolybdic acid blood filtrate (11) or ultrafiltrate were added 1 cc. of a 5 per cent alcoholic solution of the recrystallized dinitrobenzoic acid<sup>5</sup> and 1 cc. of 5 per cent NaOH. The 0.1 per cent creatinine stock solution (in 0.1 N HCl) was diluted with water to give appropriate standards (of actual concentrations of from 0.1 to 0.5 mg. per cent), and 5 cc. of the standard were treated in the same way as the filtrate. In these concentrations no blank color is given by the reagents alone and pure creatinine solutions in concentrations corresponding to from 0.5 to 1.0 mg. per 100 cc. of blood yield enough color for colorimetric readings. Samples of human whole blood were used.

<sup>6</sup> These specimens were obtained through the courtesy of our colleague, Dr. Ephraim Shorr.

Ultrafiltrates from human plasma were made with an ultrafilter of the Giemsa type and the reaction was studied as applied to 1:5 and 1:2.5 dilutions of these filtrates. A few studies were also carried out upon filtrates from beef blood and ultrafiltrates from beef

TABLE III

*Figures for Creatine in Urine of Myxedema Patient with Creatinuria and for Creatine Added to Normal Urine Determined by the New Method and by Two Picrate Methods*

Specimen	Method	Pre-formed creatinine	Total creatinine	Creatinine creatinine	Creatinine recovered
		mg. per 100 cc. urine	mg. per 100 cc. urine	mg. per 100 cc. urine	per cent
1. Normal urine* + creatine (43.4 mg. as creatinine)	Picrate, No. II	131.9	166.8	34.9	80.4
	New method	122.9	160.0	37.1	85.4
2. " "	Picrate, No. I	100.0	136.0	36.0	83.0
	" " II	99.3	142.0	42.7	98.0
	New method	99.3	136.0	36.7	84.6
3. Normal urine + creatine (26 mg. as creatinine)	Picrate, No. I	70.2	89.8	19.6	75.3
	" " II	71.9	92.9	21.0	80.8
	New method	66.2	87.9	21.7	83.4
4. Myxedema Urine I	Picrate, No. I	mg. per 24 hrs. 798.0	mg. per 24 hrs. 1180.0	mg. per 24 hrs. 382.0	
	" " II	799.0	1180.0	390.0	
	New method	790.0	1176.0	386.0	
5. " " II	Picrate, No. I	802.5	1247.0	444.5	
	" " II	812.2	1260.0	438.8	
	New method	826.5	1275.0	448.5	
6. " " III	Picrate, No. I	757.5	1095.0	337.5	
	" " II	769.5	1146.0	376.5	
	New method	750.0	1128.0	378.0	
7. " " IV	Picrate, No. I	799.5	1209.0	409.5	
	" " II	832.5	1239.0	406.5	
	New method	802.5	1209.0	406.5	

\* The normal urines contained no appreciable amount of creatine.

serum. Determinations were also made on these same filtrates by the Folin (2) and the Folin-Wu (3) picrate blood methods.

The color obtained with human blood filtrates is so different from that of creatinine solutions that accurate colorimetric read-

ings are not possible. The color from the blood filtrates is yellowish brown as compared with the rose color yielded by creatinine, and the difference in shade becomes greater after the first few minutes. Approximate colorimetric readings against creatinine standards indicated that the amount of color produced in the tungstomolybdate filtrates at the end of 1 minute is probably in the range to be expected from results by picrate methods (usually higher than the Folin-Wu figure and lower than the Folin figure), but the difference in shade of color between standard and unknown is so great that the readings obtained here probably represent little better than guesswork. The color in the filtrate fades rapidly compared with the standards and at the end of 10 minutes may appear to have decreased by as much as from 20 to 40 per cent.

In ultrafiltrates from human plasma the difference between the picrate and dinitrobenzoate values was equally great and the fading of color in the filtrates and their failure to match the standard colors by the dinitrobenzoic acid method were, if anything, more marked. Tungstomolybdate filtrates from beef blood and ultrafiltrates from beef serum also failed to match creatinine standards by the dinitrobenzoic acid method, but the divergency of color from that of the standards and the change and fading of color were much less marked than in the case of human blood.

Figures by the new method for creatinine values in the individual filtrates are not given because we feel that they would have no special significance.

The following findings indicate that the differences between blood filtrates and creatinine solutions with respect to color and rate of color development are due to the nature of the chromogenic compound present in blood and not to the presence of other substances. (a) In contrast to the blood filtrates, urine, when tested undiluted, or after dilution so that it contained an amount of creatinine corresponding to the apparent creatinine concentration in blood filtrates and treated with dinitrobenzoic acid according to the blood method, gives a color exactly like that of pure creatinine solutions. (b) To 25 cc. of tungstomolybdate filtrate from human blood were added 0.5 cc. of N oxalic acid and 100 mg. of Lloyd's reagent. After a 10 minute shaking the solution was filtered, and the filtrate neutralized with carbonate and again filtered. Such

filtrates contain most of the known constituents of blood filtrates but only traces of the chromogenic substance. Creatinine added to such filtrates gives a color with the dinitrobenzoic acid method practically identical with that of creatinine, with constant readings against creatinine standards up to at least 50 minutes. (c) Solutions containing urea, uric acid, glucose, sodium chloride, sodium sulfate, glycine, and creatinine in such amounts as might be present in a blood filtrate also match pure creatinine solutions almost perfectly by the dinitrobenzoic method and the readings against creatinine standards were constant over periods of 1 hour. Glycine, urea, and uric acid in concentrations 3 times as great as those of normal blood and glutathione and thioneine in amounts equivalent to 100 and to 20 mg. respectively per 100 cc. of blood have no effect upon the color given by creatinine in the dinitrobenzoic acid method. Sulfate and chloride have only a slight brightening effect upon the creatinine color, more noticeable during the first few minutes than later. Glucose up to 0.2 per cent of the original blood has no appreciable effect on the reaction. Oxalate in the amounts contained in our blood filtrates is without effect.

#### DISCUSSION

The question of the identity of the creatinine-like chromogenic material or materials of blood is still unanswered. Danielson (12) has recently published a paper on this subject in which he concludes that the chromogenic substance of blood is true creatinine. We may point out, however, that the net result of Danielson's work in this connection is the addition of another type of blood filtrate (ultrafiltrate) to the two forms of filtrate (heat and tungstic acid) which, as we had already shown (13), lose much or all of the creatinine chromogenic material when shaken with kaolin.<sup>7</sup> In his summation of the arguments for and against the view that the chief chromogenic compound in blood is true creatinine, Danielson omits the crucial finding which, we believe, cannot be reconciled with the conclusion that the compound is creatinine. Thus

<sup>7</sup> The failure of Danielson (contrary to our findings (13)) to observe any difference between the color developed in creatinine solutions and picric acid blood filtrates when carbonate is used as alkali is adequately explained by the fact that Danielson did not use picric acid blood filtrates in making this test.

Danielson ((12) p. 193) states, "The conclusion seems unavoidable that picric acid in the presence of blood filtrate constituents hinders the adsorption of creatinine or during the process of protein precipitation a new chromogenic material is formed which is not adsorbed by kaolin." Obviously neither of these assumptions will in any measure explain the fact (first reported by Behre and Benedict (13) and corroborated by Gaebler (14) and by Bohn and Hahn (15)) that if pure creatinine be added to fresh whole blood, and this blood be then precipitated with picric acid, kaolin will remove the added creatinine practically quantitatively, while it leaves unaffected the chromogenic material originally present in the blood. It seems to us obvious that when it is shown that creatinine added to the original blood behaves differently from the chromogenic substance in this same blood, even under only one condition, the two compounds cannot be considered one and the same substance. Similarity of behavior under any number of other conditions would not alter the validity of this conclusion. Danielson has entirely avoided the fundamental issue raised by this fact. As long as the above finding has not been disproved or is not adequately explained, we believe that no one can logically maintain that the chromogenic substance in blood can be preformed creatinine. Certainly no amount of proof that, under a multitude of conditions other than those we used, the chromogenic substance in blood may be removed by kaolin or by Lloyd's reagent will suffice to show that the chromogenic substance is creatinine, any more than such findings would show that creatinine and 2-benzylcreatinine, both of which compounds are removed from dilute acid solution by Lloyd's reagent and both of which react very similarly with alkaline picrate, are one and the same compound. Danielson, like Hayman, Johnston, and Bender, has adopted the reasoning which seems to us entirely fallacious, that because two substances behave similarly in a number of instances these compounds are one and the same, in spite of the fact that conditions may be found under which the compounds present in the same solution (original blood after addition of creatinine) can be sharply separated from one another. As we have pointed out in a previous paper (16), this type of reasoning would readily identify  $\alpha$ - and  $\beta$ -glucose as one and the same compound, so long as results with the polariscope were disregarded.

In the present paper we have presented additional evidence in favor of the view that the preformed chromogenic compound in blood is not creatinine. This is the finding that filtrates from human blood fail, under the conditions of our procedure, to yield, with alkali and 3,5-dinitrobenzoic acid, a color reaction which resembles at all closely that given by pure creatinine solution, by creatinine added to synthetic blood filtrates, or by creatinine added to blood filtrates from which the chromogenic substance has been removed by Lloyd's reagent. We recognize that this finding does not constitute final proof that the preformed chromogenic material in blood is not creatinine, but we believe that it offers strong corroborative evidence for such a view. Color reactions are subject to changes in intensity and in shade of color as the result of the presence of various compounds which by themselves may apparently not react with the reagent, and there is a possibility that such an "interfering" compound may be present in blood, and that this substance may be removed by Lloyd's reagent, together with the chromogenic compound. That such an explanation of our results is correct is not only inherently unlikely, but is rendered very highly improbable by the finding that urine, which contains most if not all of the compounds present in blood, presents no difficulty in the determination of creatinine with the new reagent, either by the technique which we recommend above for this purpose or, after dilution, by the technique which has been applied to blood filtrates.

We believe that an impartial consideration of the facts at present available concerning the behavior of the blood chromogenic compound as compared with the behavior of true creatinine necessitates the conclusion that these compounds are not one and the same. How easily and under what conditions the chromogenic, or some other compound in blood may be converted into creatinine, are questions which remain to be answered in the future.

In conclusion, we may point out that our study of the nature of the reaction between 3,5-dinitrobenzoic acid and creatinine has so far been very limited. We have obtained evidence of the existence of one and probably of two compounds of dinitrobenzoic acid and creatinine, but our work in this connection is very incomplete.

## SUMMARY

A new color reaction for creatinine with 3,5-dinitrobenzoic acid in presence of alkali is described. Results obtained by the application of this reaction to various creatinine derivatives, to glycoxyamidine and hydantoin, and to urine and blood filtrates are described. A method for the determination of urinary creatinine by the new reagent is described. Determinations made upon human urine samples from which creatinine has been removed by Lloyd's reagent indicate that figures for urinary creatinine by the picrate methods are from 2.5 to 5 per cent too high. Human blood filtrates yield with the dinitrobenzoic acid method a color so different, both in shade and stability, from that given by creatinine that the chromogenic substance in such filtrates cannot be determined as creatinine. The significance of these results in relation to the question of the creatinine chromogenic material of blood is discussed. The findings are interpreted as furnishing further evidence in favor of the view that this chromogenic material is not creatinine.

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## THE SYNTHESIS OF DJENKOLIC ACID\*

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The possibility of the existence in nature of unknown sulfur-containing amino acids has received much attention. The announcement of the isolation of djenkolic acid, a sulfur-containing amino acid, from the djenkol bean (*Pithecolobium lobatum*) by Van Veen and Hyman (1) is, therefore, of considerable interest. It is possible that the compound may be more widely distributed in nature and perhaps may be responsible for a portion of the non-cystine, non-methionine sulfur in certain proteins, particularly those of other leguminous plants.

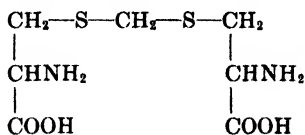
The structure assigned to the compound by these investigators is also of considerable interest because of its striking relationship to cysteine. The suggested structure would indicate that djenkolic acid is the cysteine thioacetal of formaldehyde. The synthetic approach to such a thioacetal by the methods generally available is beset with numerous difficulties. Having in our hands, however, a method, which has been useful in the synthesis of thio ethers and which we felt could be extended to the synthesis of formaldehyde thioacetals, we have undertaken the synthesis of djenkolic acid in order to afford final proof of the structure of the compound and in addition to make it more readily available for physiological study. The isolation of the compound in pure form from natural sources, as indicated by Van Veen and Hyman, is very difficult.

\* Since Van Veen and Hyman had indicated that they were engaged in the synthesis of djenkolic acid, and not wishing to anticipate the publication of a successful synthesis from their hands, we have communicated with Dr. Van Veen. He has informed us that owing to pressure of other work they were unable to continue their synthetic attack and has urged us to proceed with the presentation of our work. We have forwarded a sample of the synthetic compound to his laboratory. We wish to express our appreciation for the very friendly cooperation of Dr. Van Veen.



Curiously enough the djenkolic acid was first isolated from the urine of natives of Java who had eaten the djenkol bean and were suffering from djenkol poisoning. It was later that the compound was isolated from the djenkol bean itself. As stated by Van Veen and Hyman, the natives of Java relish the djenkol bean as an article of food, although a severe poisoning may result from its ingestion. Apparently individuals differ greatly in their sensitivity, some becoming sick after eating as little as one-half of a bean weighing about 15 gm., while others may eat up to ten or more beans before the occurrence of pathological symptoms such as abdominal and lumbar pains accompanied by painful micturition. The urine of such individuals usually contains many erythrocytes, leucocytes, epithelial cells, and coagulable protein. It was also noticed that the urine contained many small sharp difficultly soluble crystals. Suspecting some relation of the crystals to the pathological condition, Van Veen and Hyman isolated the crystals from the urine and then succeeded in isolating the same crystals from the djenkol beans. The latter was accomplished after the beans had been hydrolyzed with  $\text{Ba}(\text{OH})_2$  at  $30^\circ$  for a prolonged period of time.

The ultimate analysis of the purified material indicated an empirical formula of  $\text{C}_7\text{H}_{14}\text{O}_4\text{N}_2\text{S}_2$ . A positive ninhydrin reaction and the amphoteric properties of the compound suggested that they were dealing with an amino acid. The sulfur was readily split out as  $\text{PbS}$  with alkali and lead acetate, but, since the nitroprusside test was negative before and after reduction, it was suspected that the compound was a thio ether. Their real clue to the structure came through the isolation of cystine after treatment of the compound with concentrated  $\text{H}_2\text{SO}_4$ . This reaction is quite analogous to the formation of homocystine by Butz and du Vigneaud (2) through the action of 18 N  $\text{H}_2\text{SO}_4$  on methionine. Since no methyl group was found to be attached to the sulfur or to the nitrogen, Van Veen and Hyman decided that a methylene group must be between the sulfurs of two cysteine radicals. The compound would, therefore, have the following structure:



In support of this view they were able to identify formaldehyde as one of the split-products from the reaction with  $\text{H}_2\text{SO}_4$ . In addition they were able to prepare a dibenzoyl and a dihydantoin derivative as well as a monohydrochloride, the analyses of which corresponded to the compounds indicated by their hypothesis.

In considering the suggested structure of djenkolic acid it occurred to us that perhaps methylene chloride would react in liquid ammonia with 2 molecules of cysteine to yield djenkolic acid, just as we had found that methyl iodide reacted with cysteine to yield methyleysteine (3) and with homocysteine to yield methionine (4). Accordingly cystine was dissolved in liquid ammonia, reduced with metallic sodium, and to the liquid ammonia solution methylene chloride was added. After evaporation of the ammonia the residue was taken up in water and the reaction of the solution was adjusted so that it was acid to litmus but alkaline to Congo red. The condensation product crystallized in needles. After recrystallization from a large volume of water the product gave a negative nitroprusside test for the sulfhydryl grouping, both before and after treatment with cyanide. The analytical values of the compound and other properties indicated that we had obtained the desired compound, the cysteine thioacetal of formaldehyde.

The synthetic compound possesses the properties described for djenkolic acid, although the optical rotation is higher than that reported by Van Veen and Hyman for the isolated product. The synthetic compound has a specific rotation of  $-44.5^\circ$ , while Van Veen reported a rotation of  $-25^\circ$ . This is not surprising, as pointed out by Van Veen in a private communication to us, because opportunity for racemization is undoubtedly afforded in the process of isolation of the djenkolic acid from the djenkol beans, particularly in the  $\text{Ba}(\text{OH})_2$  treatment.

The other properties of the compound, however, correspond closely to those of the naturally occurring compound. It decomposes in the same range of temperature,  $300-350^\circ$ , as reported by Van Veen and Hyman. It has also the same general solubility in water and acid. It likewise yields  $\text{PbS}$  under the same conditions as those employed by Van Veen and Hyman for djenkolic acid, and through the action of concentrated  $\text{H}_2\text{SO}_4$  cystine is formed. The benzoyl derivative and the hydrochloride of the synthetic product also possess the chemical behavior and physical properties reported

for the corresponding derivatives of the naturally occurring compound.

The comparison of the above properties of the synthetic cysteine thioacetal of formaldehyde and those of the naturally occurring djenkolic acid affords convincing evidence that the two substances are identical.

#### EXPERIMENTAL

*Preparation of the L-Cysteine Thioacetal of Formaldehyde (Djenkolic Acid)*—23 gm. of cystine were added to about 400 cc. of liquid ammonia in a three-neck flask fitted with a mechanical stirrer and a soda-lime tube. The flask was immersed in a bath of trichloroethylene containing solid  $\text{CO}_2$ . Sodium was introduced into the flask in small portions until a blue color, indicating an excess of sodium, persisted for 3 minutes; about 9 gm. of sodium were used. 23 cc. of methylene chloride were then added, after which the ammonia was allowed to evaporate. The residue was treated with 250 cc. of water and the solution was filtered through a thin layer of norit to remove a slight turbidity. Neutralization of this solution with HCl gave a precipitate which was filtered and was washed with 800 cc. of ice water in small portions. At this stage the wash liquid gave a negative sulfhydryl test with nitroprusside, but the residue yielded a slightly positive reaction. The product was dissolved in 200 cc. of water containing enough HCl to give complete solution. The compound was reprecipitated by neutralization, and was washed with ice water until the wash liquor gave negative sulfhydryl and disulfide tests. The crystalline compound itself now gave a negative sulfhydryl test before and after treatment with NaCN. The dry product weighed 7.9 gm., considerable material having been sacrificed in getting the material sulfhydryl- and disulfide-free. Considerably higher yields can be obtained by reworking the mother liquors. The compound crystallized in small rosettes of needles, as described by Van Veen and Hyman for djenkolic acid. The compound melted at  $300\text{--}350^\circ$  with previous darkening, and possessed a rotation of  $[\alpha]_D^{20} = -44.5^\circ$  for a 2 per cent solution in 1 per cent HCl. The product had the following composition.

3.918 mg. substance: 4.74 mg.  $\text{CO}_2$  and 1.95 mg.  $\text{H}_2\text{O}$

3.514 " " : 0.342 cc. N at  $29.5^\circ$  and 765 mm.

5.635 " " : 10.448 mg.  $\text{BaSO}_4$  (micro-Carius)

$\text{C}_7\text{H}_{14}\text{O}_4\text{N}_2\text{S}_2$ . Calculated. C 33.04, H 5.55, N 11.02, S 25.22

Found. " 33.00, " 5.57, " 11.05, " 25.47

The hydrochloride of the synthetic compound was prepared as described by Van Veen and Hyman for the hydrochloride of djenkolic acid. The above compound was dissolved in dilute HCl and the solution was concentrated *in vacuo*. The monohydrochloride separated in plates which corresponded in crystalline form to the description of the hydrochloride of djenkolic acid given by Van Veen and Hyman. The recrystallized material gave the following analysis.

5.112 mg. substance: 2.504 mg. AgCl

$C_7H_{11}O_4N_2S_2Cl$ . Calculated, Cl 12.20; found, Cl 12.12

*Preparation of the Dibenzoyl Derivative*—The directions of Van Veen and Hyman ((1) 1935) were followed. 0.25 gm. of the thioacetal was dissolved in 10 cc. of 2 N NaOH and the solution cooled in an ice bath. Then 1.5 cc. of benzoyl chloride were added in small portions, with vigorous shaking. After standing in the ice box overnight the reaction mixture was acidified with dilute  $H_2SO_4$  and filtered. The crude product was extracted repeatedly with boiling benzene, and was then dissolved in 10 cc. of methyl alcohol. An equal volume of water was added and upon slow evaporation of the solution silky needle-like crystals formed. These were twice recrystallized from 50 per cent methyl alcohol and air-dried. The product formed a milky liquid at  $86^\circ$  with previous softening, as described for dibenzoyldjenkolic acid. This material lost its water of crystallization on drying at  $110^\circ$  over  $P_2O_5$  *in vacuo*. The anhydrous material had the following composition.

4.238 mg. substance: 0.232 cc. N at  $28^\circ$  and 775 mm.

$C_{21}H_{22}O_6N_2S_2$ . Calculated, N 6.06; found, N 6.33

The authors wish to thank Mr. C. Rodden, microanalyst of this laboratory, for carrying out the microanalyses.

#### SUMMARY

The cysteine thioacetal of formaldehyde has been synthesized and has been shown to agree closely in physical properties and chemical behavior with the djenkolic acid isolated by Van Veen and Hyman from natural sources.

The hydrochloride and benzoyl derivatives have been prepared

and likewise exhibit the properties of the corresponding derivatives of djenkolic acid.

*Addendum*—After this paper had been submitted for publication, we received a highly purified sample of the naturally occurring djenkolic acid from Dr. Van Veen, for which we wish to express our appreciation. This has enabled us to make a direct comparison between our synthetic compound and the naturally occurring substance. The sample of the djenkolic acid which we received possessed the same rotation as our synthetic compound, namely  $-44.5^\circ$ . Both compounds possessed the same crystalline form and decomposition point. Their hydantoins melted at the same temperature and a mixture of the two showed no depression of the melting point. It was found that the synthetic compound and the naturally occurring djenkolic acid had the same solubility in water, namely 1.02 gm. per liter at  $30^\circ \pm 0.5^\circ$ . Furthermore, the naturally occurring compound was added to a saturated solution of the synthetic material in contact with its solid phase; no change in the solubility occurred, thus demonstrating beyond question the identity of the two products.

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# REAGENTS FOR THE ISOLATION OF CARBONYL COMPOUNDS FROM UNSAPONIFIABLE MATERIAL\*

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In a recent publication (1) it was shown that in all probability the ketones cholestenone and coprostanone are intermediate products of cholesterol metabolism.<sup>1</sup> This cannot be regarded as proved, however, until these substances have been isolated from unsaponifiable material of animal origin. It is to be expected that such intermediate products would occur only in very small amounts. The isolation of small amounts of such ketones from organs, with the methods available, would present great difficulty.

The reagents which we investigated have the following main characteristics: They possess besides an  $\text{—NH} \cdot \text{NH}_2$  or an  $\text{—ONH}_2$  group, a free carboxyl group, so that they form with the ketones, acidic hydrazones or oximes. The alkali salts of these acids are water-soluble, and therefore may be quantitatively separated from the ethereal solution of the other unsaponifiable material. On acidification, the insoluble hydrazone acids or oxime acids are precipitated and can be filtered off or extracted with ether. The ketones can be readily regenerated from them.

The following substances were used.

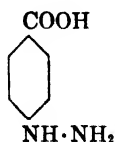
1. *p*-Carboxyphenylhydrazine (*p*-hydrazinobenzoic acid).

\* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

This report is from a thesis submitted by M. Anchel in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

<sup>1</sup> The presence of cholestenone is further suggested by the fact that unsaponifiable material from arteriosclerotic aortas gives an absorption spectrum characteristic for  $\alpha, \beta$ -unsaturated ketones (2).

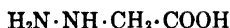
2. Carboxymethoxylamine (aminooxyacetic acid).<sup>2</sup>
3. Carboxymethylhydrazine (hydrazinoacetic acid).



(1)



(2)



(3)

With a view to using these reagents for the isolation of keto derivatives of cholesterol, their behavior toward the ketones cholestanone, coprostanone, and cholestenone was investigated.

*p*-Carboxyphenylhydrazine, which has been employed (3) for the detection of pyruvic acid, proved to be of particular value for our purpose, in that it enabled us to separate cholestenone from the saturated ketones. While the hydrazones of the saturated ketones are split by refluxing in alcoholic formaldehyde solution, the hydrazone of the  $\alpha,\beta$ -unsaturated ketone, cholestenone, is not attacked under these conditions. This ketone can be recovered from its hydrazone by refluxing in an alcoholic solution of pyruvic acid.

We are not yet certain as to the explanation for the difference in behavior of the hydrazones of the saturated and unsaturated ketones.<sup>3</sup> We have reason to believe that the hydrazones of  $\alpha,\beta$ -unsaturated ketones are in general more difficultly split than those of the saturated ketones.

The working up of a mixture of mineral oil, cholesterol, cholestanone, and cholestenone is described below.

The *p*-carboxyphenylhydrazones are autoxidizable, and their isolation must therefore be carried out, as far as possible, in the absence of air. The oxime acids are not attacked by air. The first step in working up unsaponifiable material would therefore be the isolation of total ketones in the form of their oxime acids. After the regeneration of the ketones by refluxing with hydrochloric acid, *p*-carboxyphenylhydrazine can be used to separate

<sup>2</sup> The use of this reagent was suggested to us by Professor H. T. Clarke.

<sup>3</sup> The cholestenone compound is not a pyrazoline, since these are not attacked by pyruvic acid.

the saturated ketones from the  $\alpha,\beta$ -unsaturated ketone, cholestenone.

Carboxymethylhydrazine is not as satisfactory as the other reagents. It reacts only slowly with cholestenone, and the hydrazone formed is not readily extracted from the ether. As in the case of cholestenone *p*-carboxyphenylhydrazone, the hydrazone is not attacked by formaldehyde, but can be split with pyruvic acid. With cholestanone the reagent behaves in a more satisfactory manner. The hydrazone forms almost quantitatively, and is fairly readily extracted from the ether. On acidification of the alkaline extract, the cholestanone is rapidly regenerated.

The ketone reagent,  $(\text{CH}_3)_3\text{N}-\text{CH}_2\text{CO}\cdot\text{NH}\cdot\text{NH}_2$ , recently  
 $\begin{array}{c} | \\ \text{Cl} \end{array}$

described by Girard, in a patent note (4), has the disadvantage, for our purpose, that the compound formed with cholestanone splits spontaneously in aqueous solution, making separation difficult.

The reagents described may have a further field of application in the isolation of various sex hormones. The behavior of *p*-carboxyphenylhydrazine toward  $\alpha,\beta$ -unsaturated ketone hormones (progesterone, testosterone) should be of particular interest. The reagents may also be used for the determination and isolation of keto acids. After esterification of the total acids, the mixed esters are treated as the unsaponifiable material. We are at present attempting the isolation of keto bile acids.

#### EXPERIMENTAL

*Reactions with Carboxymethoxylamine*—This reagent was first described by Werner (5). It was prepared according to the method of Borek and Clarke (6). M.p. 151°.

*Cholestanone Carboxymethoxime*—500 mg. of cholestanone were refluxed for 1 hour in 15 cc. of 90 per cent alcohol with 325 mg. of carboxymethoxylamine semihydrochloride and 500 mg. of crystalline sodium acetate. The solution was distributed between 1 per cent aqueous  $\text{K}_2\text{CO}_3$  and ether. The aqueous extract was acidified to Congo red with  $\text{HCl}$ , and the precipitate was filtered off. Yield 581 mg. (98 per cent of the theoretical).



After recrystallization from ethyl acetate, the melting point was 151–152° (corrected) with decomposition.

*Analysis*— $C_{29}H_{49}O_3N$

Calculated. C 75.75, H 10.75, N 3.05

Found. " 75.10, " 10.10, " 3.06

The cholestanone was recovered by refluxing 500 mg. of the compound in 17 cc. of 95 per cent alcohol with 3 cc. of 7 per cent HCl for 2 hours. The solution was distributed between 1 per cent  $K_2CO_3$  and ether. From the ether extract were recovered 400 mg. of crude cholestanone (95 per cent of the theoretical). After recrystallization from alcohol, the melting point was 128–129°.

*Cholestenone Carboxymethoxime*—The procedure was exactly the same as with cholestanone. Yield, 99 per cent of the theoretical. M.p. 158–159° (corrected) with decomposition.

*Analysis*— $C_{29}H_{47}O_3N$

Calculated. C 76.09, H 10.36, N 3.06

Found. " 75.59, " 10.35, " 3.17

On splitting, 94 per cent of the cholestenone was recovered. After recrystallization it melted at 80–81°.

*Coprostanone Carboxymethoxime*—The procedure was the same as with cholestanone. Yield, 97 per cent of the theoretical. M.p. 150–151° (corrected) with decomposition.

*Analysis*— $C_{29}H_{49}O_3N$

Calculated. C 75.75, H 10.75, N 3.05

Found. " 75.67, " 10.58, " 2.95

On splitting, 100 per cent of the coprostanone was recovered.

*Separation of Cholestanone from Mineral Oil by Use of Carboxymethoxylamine*—A mixture of 5 gm. of paraffin oil, 500 mg. of cholesterol, and 250 mg. of cholestanone was refluxed for 3 hours in 20 cc. of 95 per cent alcohol, with 165 mg. of reagent and 250 mg. of crystalline sodium acetate. 297 mg. of oxime acid (100 per cent of the theoretical) were obtained, which after splitting with HCl yielded 95 per cent of the cholestanone.

*Reactions with p-Carboxyphenylhydrazine*—"p-Hydrazinobenzoic acid" obtained from the Eastman Kodak Company was further purified as follows: 10 gm. were dissolved in 200 cc. of 3.5 per cent

HCl by heating. The solution was boiled with charcoal; to the hot filtrate was added a saturated solution of sodium acetate, until it was no longer acid to Congo red. It was cooled rapidly, and the reagent was filtered off under a stream of  $\text{CO}_2$ .

*Cholestanone p-Carboxyphenylhydrazone*—A solution of 500 mg. of cholestanone in 20 cc. of 95 per cent alcohol was refluxed for 2 hours with 500 mg. of reagent and a few drops of acetic acid. The mixture was distributed between 4 per cent  $\text{K}_2\text{CO}_3$  and ether. The aqueous extract was acidified to Congo red with HCl, and the precipitate was filtered off under a stream of  $\text{CO}_2$ . Yield 536 mg. (81 per cent of the theoretical).

The hydrazone was purified by dissolving it in a solution of chloroform and 95 per cent alcohol. When the chloroform was distilled off, the substance crystallized from the alcohol in hexagonal plates. These had an indefinite decomposition point above  $200^\circ$ .

*Analysis*— $\text{C}_{24}\text{H}_{32}\text{O}_2\text{N}_2$

Calculated. C 78.40, H 10.07, N 5.38

Found. " 78.04, " 9.83, " 5.02

*Cholestenone p-Carboxyphenylhydrazone*—The procedure was exactly as above. Yield, 94 per cent of the theoretical; indefinite decomposition point above  $200^\circ$ .

*Analysis*— $\text{C}_{24}\text{H}_{30}\text{O}_2\text{N}_2$

Calculated. C 78.70, H 9.72, N 5.40

Found. " 78.32, " 9.65, " 5.58

*Coprostanone p-Carboxyphenylhydrazone*—With the above procedure the yield was only 58 per cent of the theoretical. When the proportion of reagent was doubled, a yield of 87 per cent was secured. The product had an indefinite decomposition point above  $200^\circ$ .

*Analysis*— $\text{C}_{24}\text{H}_{30}\text{O}_2\text{N}_2$

Calculated. C 78.40, H 10.07, N 5.38

Found. " 77.83, " 9.94, " 5.33

*Splitting p-Carboxyphenylhydrazones of Cholestanone and Coprostanone*—500 mg. of the hydrazone were refluxed for 5 hours in 15 cc. of 95 per cent alcohol containing 0.25 cc. of aqueous 37 per cent

formaldehyde solution. The mixture was distributed between 4 per cent  $K_2CO_3$  and ether. The ethereal solutions yielded 89 per cent of the cholestanone and 91 per cent of the coprostanone, respectively.

*Splitting Cholestenone p-Carboxyphenylhydrazone*—When the hydrazone of cholestenone was treated as above, no free ketone was obtained. Only when the formaldehyde concentration was increased 20-fold, was a small percentage of the hydrazone split.

The splitting with pyruvic acid was carried out as follows: 500 mg. of the hydrazone were refluxed for 4 hours in 20 cc. of 95 per cent alcohol with 2 cc. of pyruvic acid. The solution was distributed between 4 per cent  $K_2CO_3$  and ether. From the ether extract were obtained 290 mg. (78 per cent) of cholestenone which melted at  $80-81^\circ$  after recrystallization.

*Reactions with Carboxymethylhydrazine*—The reagent was prepared according to the method of Darapsky and Prabhakar (7).

*Cholestanone and Carboxymethylhydrazine*—150 mg. of cholestanone were refluxed for 2 hours in 6 cc. of 80 per cent alcohol with 100 mg. of reagent. The mixture was distributed between ether and 1 per cent  $K_2CO_3$ . The aqueous extract was acidified to Congo red with HCl. Precipitation began immediately and appeared to be complete after 24 hours. The precipitate (130 mg.) consisted of cholestanone. M.p.  $128-129^\circ$  after recrystallization. A further 20 mg. of cholestanone were obtained from the ethereal solution. Attempts to isolate the unsplit hydrazone from the aqueous extract were unsuccessful.

*Cholestenone and Carboxymethylhydrazine*—150 mg. of cholestenone were treated in the same way as described for cholestanone. The aqueous extract on acidification gradually became cloudy. After 5 days the precipitate was filtered off. It was obviously a mixture; two such preparations contained 4.57 per cent and 3.86 per cent of nitrogen, respectively (theory, 6.14). The product was not attacked on boiling with an alcoholic solution of formaldehyde. It was, however, split by alcoholic pyruvic acid. 30 mg. of the compound were refluxed for 2 hours in 3 cc. of 95 per cent alcohol with 0.5 cc. of pyruvic acid, and the solution distributed between ether and 1 per cent  $K_2CO_3$ . The ethereal solution yielded 18 mg. of cholestenone (72 per cent of the theoretical).

*Separation of Saturated and Unsaturated Ketones from Mineral Oil by Use of p-Carboxyphenylhydrazine*—A mixture of 15 gm. of medicinal paraffin oil, 500 mg. of cholestanone, 500 mg. of cholestenone, and 1 gm. of cholesterol was refluxed for 4½ hours in 40 cc. of 95 per cent alcohol, with 1.45 gm. of reagent. The reaction mixture was distributed between ether and 4 per cent  $K_2CO_3$ . The aqueous extract was acidified to Congo red with HCl, and the hydrazones were filtered off under a stream of  $CO_2$ . Yield 1.22 gm. (92 per cent of the theoretical).

To 500 mg. of the above hydrazone mixture in 15 cc. of 95 per cent alcohol, 0.5 cc. of 37 per cent formaldehyde was added. After being refluxed for 5 hours, the products were distributed between 4 per cent  $K_2CO_3$  and ether. The ethereal solution yielded 364 mg. (98 per cent of the theoretical) of ketone, which after recrystallization melted at 128–129° and showed no depression with cholestanone.

The aqueous extract yielded 575 mg. of a mixture of the hydrazones of cholestenone and formaldehyde. Of this, 500 mg. were refluxed for 3 hours in 15 cc. of 95 per cent alcohol, with 2 cc. of pyruvic acid. The solution was distributed between ether and 4 per cent  $K_2CO_3$ . From the ether there were obtained 248 mg. of ketone (87 per cent of the theoretical). M.p. 80–81° after recrystallization; no depression with cholestenone.

#### SUMMARY

1. For the isolation of ketones from unsaponifiable material, reagents were employed which possess, besides an  $-NH \cdot NH_2$  or an  $-ONH_2$  group, a free carboxyl group. These reagents react with the ketones to form acidic hydrazones or oximes which can be quantitatively separated from the residual material by means of their water-soluble alkali salts.

2. The behavior of *p*-carboxyphenylhydrazine, carboxymethylamine, and carboxymethylhydrazine toward the ketones cholestanone, cholestenone, and coprostanone is described in detail.

3. The carboxyphenylhydrazones of the saturated ketones are split by alcoholic formaldehyde; that of the  $\alpha,\beta$ -unsaturated ketone, cholestenone, is not attacked under these conditions, but

can be split by alcoholic pyruvic acid. By taking advantage of this difference in behavior, cholestenone can be separated quantitatively from the saturated ketones.

The authors are grateful to Professor Hans T. Clarke for many valuable suggestions.

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## A POTENTIOMETRIC ADAPTATION OF THE SHAFFER-HARTMANN SUGAR METHOD\*

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Wood (1) and Shaffer and Williams (2) have recently shown that sugars can be quantitatively determined by reduction of alkaline ferricyanide reagents followed by potential measurements on the solutions. The latter authors found that exceedingly minute amounts of sugar can be determined by such a method. The technique is simple and rapid. Since copper sugar reagents are generally more selective in their oxidation than the ferricyanide reagents, it seemed that a potentiometric adaptation of a copper reduction method might be worked out which would combine the advantages of both procedures. The writers believe they have succeeded in doing this.

The method consists in heating the sugar solution with Shaffer-Somogyi Reagent 50 (3) and then adding a ferricyanide-citrate<sup>1</sup> solution and measuring the potential against a blank treated similarly. The cuprous oxide is oxidized by the ferricyanide and the reaction goes essentially to completion as a result of cupric ions being removed by the high concentration of citrate. The potentials observed are close to the theoretical values calculated from the change in ferricyanide to ferrocyanide ratio occasioned by complete oxidation of the cuprous ions formed in the reduction.

### *Reagents—*

1. Shaffer-Somogyi Reagent 50. The iodate may be omitted.
2. Ferricyanide-citrate reagent. 50 gm. of  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$  (we used Merck's C.P. grade) are dissolved in warm water in a 100 cc. volumetric flask, with care that the final volume does not ex-

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<sup>1</sup> Tartrate and oxalate were less satisfactory.

ceed 85 to 90 cc. After cooling, 0.7407 gm. of  $K_3Fe(CN)_6$  and 0.1056 gm. of  $K_4Fe(CN)_6 \cdot 3H_2O$  are added and dissolved.<sup>2</sup> The solution is then brought to the mark and mixed. The final concentrations are: citrate 1.7 M, ferricyanide 0.0225 M, ferrocyanide 0.0025 M; ferricyanide to ferrocyanide = 9, as used by Shaffer and Williams. The potential of a sample of the reagent (preserved in a brown bottle in the dark) against a 0.05 M acid phthalate quinhydrone electrode was unchanged after 1 week, was 1.00 millivolt low after 2 weeks, and 2.00 millivolts low after 3 weeks. Such changes are of small consequence, since the reagent is used in both the reference and sample electrodes.

*Apparatus*—Potentials were measured with a Leeds and Northrup Students' type potentiometer, with platinum electrodes. The blank tube and sugar tube were connected with a saturated KCl-agar bridge cut off a bit after each determination. A Leeds and Northrup enclosed lamp and scale galvanometer was used.

### *Procedure*

Sugar solutions and water blanks are heated with the copper sugar reagent in the usual way as described by Shaffer and Somogyi (3). After heating, the rack containing the tubes is placed in a water bath at 20–25° and 5 cc. of the ferricyanide-citrate reagent, accurately measured, are added as soon as possible to each tube with shaking. After a few minutes with occasional shaking the cuprous oxide is all dissolved and the solutions become emerald-green in color. The tubes are then brought to 25° in a water bath<sup>3</sup> and the potentials of the sugar solutions are read against the blanks. Sugar values are taken from a curve plotted with data obtained on known sugar solutions. Table I shows potential readings on 0.02 to 2.0 mg. of sugar per 5 cc. of solution and also the theoretical potentials calculated from the change in ferricyanide to ferrocyanide ratio occasioned by the cuprous ions formed in the reductions. It will be seen that there is satisfactory agreement between the observed and calculated potentials.

There is a small amount of  $Cu^+$  formed in both the blanks and sugar solutions by autoreduction of the copper reagent. This obviously changes the ferricyanide to ferrocyanide ratio in the

<sup>2</sup> C.P. or reagent grades were used.

<sup>3</sup> We have found regulation to 0.5° satisfactory.

blanks to less than the theoretical value of 9 and supposedly the ratios in the sugar solutions are proportionately affected. The theoretical calculations are corrected for this effect.

This was done by determining the E.M.F. of a cold blank measured against a heated blank after the addition of the ferricyanide-citrate reagent to both. An E.M.F. of 2 millivolts was found which corresponds to about 0.001 mM of  $\text{Cu}^+$  formed by autoreduction of the copper reagent. This value was subtracted from the ferricyanide and added to the ferrocyanide concentrations in making the theoretical calculations.

TABLE I

*Change in Potentials of Sugar Solutions Due to Formation of Cuprous Ions in Reductions*

Glucose in 5 cc. solution mg.	E.M.F. observed	E.M.F. calculated
2.00	0.0925	0.0923
1.50	0.0692	0.0688
1.00	0.0500	0.0502
0.75	0.0400	0.0408
0.50	0.0295	0.0306
0.25	0.0170	0.0180
0.10	0.0080	0.0083
0.05	0.0040	0.0044
0.02	0.0020	0.0018

#### *Determination of Sugar in Blood and Urine Filtrates*

Potentiometric determinations on zinc (4), tungstate, and iron<sup>4</sup> (5) filtrates of blood, together with values found by iodometric titration, are shown in the tabulation below. The results represent determinations on filtrates of the same blood.

Filtrate	Glucose		
	Iron	Zinc	Tungstate
	mg.	mg.	mg.
Potentiometric.....	105, 107, 108	99, 101, 101	126, 125, 125
Titrimetric.....	105, 105	100, 100	134, 135

<sup>4</sup> After neutralizing with  $\text{BaCO}_3$  and filtering, the filtrate was made just acid to Congo red with a drop of  $\text{H}_2\text{SO}_4$  and filtered. 5 cc. portions were



The agreement between the two methods on iron and zinc filtrates is satisfactory, though zinc filtrates give somewhat better potential readings. Shaffer and Williams found zinc filtrates preferable for their procedure.

Potentiometric and titration reduction values on a ferric sulfate-Lloyd's reagent filtrate of urine<sup>5</sup> (6) were as follows, calculated as mg. of glucose per 100 cc. of urine: potentiometric, 58, 58, 59; titrimetric, 55, 57, 56.

The potentiometric method here described should be applicable to any of the common reducing sugars when used with the appropriate Shaffer-Somogyi reagent.

#### SUMMARY

A potentiometric adaptation of the Shaffer-Hartmann sugar method has been developed. It has been found applicable to pure sugar solutions, iron and zinc filtrates of blood, and ferric sulfate-Lloyd's reagent filtrates of urine.

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pipetted into sugar tubes and drops of 0.5 N NaOH were added to neutralize to phenol red (the amount required was determined on another sample plus indicator). The copper reagent was then added and the procedure continued as usual.

<sup>5</sup> The trace of barium was removed from this filtrate with a drop of H<sub>2</sub>SO<sub>4</sub>, as in the case of the iron filtrate of blood (foot-note 4). The samples were measured and treated similarly to the blood filtrate samples.

# DETERMINATION OF CHLORIDES IN BIOLOGICAL FLUIDS BY THE USE OF ADSORPTION INDICATORS

## THE USE OF DIPHENYLAMINE BLUE FOR THE VOLUMETRIC MICRODETERMINATION OF CHLORIDES IN URINE AND BLOOD FILTRATES

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The increasing importance of adsorption indicators for micro-volumetric work is evidenced by the large number of papers which have appeared in the literature during the past few years (1-3). The authors, in continuing their work on the application of adsorption indicators to the determination of chlorides in biological fluids (4), have found that, with slight modifications, dichloro-fluorescein could be used as an adsorption indicator for chloride determinations in practically all neutral biological fluids, *e.g.* plasma,<sup>1</sup> pleural fluids,<sup>2</sup> synovial fluids,<sup>2</sup> etc. Although practically every adsorption indicator mentioned in the literature and a great many dyes were tried out in both neutral and strongly acid media, dichlorofluorescein apparently gave the best results for chloride determinations in a neutral medium, while diphenylamine blue proved to be best for a strongly acid medium.

For the determination of chlorides in urine and blood filtrate it was found necessary to work in strongly acid medium to prevent the precipitation by the silver nitrate of phosphates, urates, tungstates, etc. Lang and Messinger (5), using diphenylamine blue

<sup>1</sup> For plasma, the same procedure is carried out as in the previous paper (4) except that 7 ml. of the alcohol-ether solution (3:1) are used. As an improvement in the original procedure, better results are obtained if the alcohol-ether filtrate is transferred to another tube before titrating.

<sup>2</sup> For pleural and synovial fluids the same procedure is followed as for blood serum (4).

as an adsorption indicator, established a method for the determination of 15 mg. to 170 mg. of  $\text{Cl}^-$  in pure chloride solutions which can be as much as 5 N with respect to sulfuric, nitric, perchloric, hydrofluoric, and oxalic acids. The reverse titration also gives satisfactory results.

The authors have succeeded in applying this method to urine and blood filtrates on a microscale for the determination of quantities in the range of 1 mg. of sodium chloride. The indicator, however, is prepared as an integral part of the determination and caprylic alcohol is used to give a sharper end-point by clarifying the solution at the equivalence point. The advantages of this method over the Volhard are that the results obtained are just as accurate and capable of better reproducibility, only one standard solution (sodium chloride) is necessary, the presence of the silver chloride is a necessary part of the determination, the solution is titrated directly with the silver nitrate, and the end-point is sharp and fades only after the solution has stood for some time. The advantage over the method described by Fearon and Gillespie (1) is that urine can be titrated directly with the silver nitrate, while these authors use the reverse process with tartrazine as the indicator.

#### EXPERIMENTAL

##### *Solutions—*

Standard sodium chlorides. A 0.1 N solution of NaCl was prepared by dissolving 5.846 gm. of dry c.p. NaCl in a liter of solution. 1 ml. is equivalent to 5.846 mg. of NaCl.

Standard silver nitrate. An approximately 0.02 N solution of  $\text{AgNO}_3$  was made by diluting a 0.1 N solution of  $\text{AgNO}_3$ . The standardization was performed as follows: 0.2 ml. of 0.1 N NaCl was pipetted into a test-tube of convenient size ( $6 \times \frac{3}{4}$  inches); 4 ml. of 5 N  $\text{H}_2\text{SO}_4$ , 4 drops of the indicator, and 0.2 ml. of 0.1 N  $\text{K}_2\text{Cr}_2\text{O}_7$  were added. The solution was allowed to stand for a minute and 5 drops of caprylic alcohol were added. The samples were then titrated with 0.02 N  $\text{AgNO}_3$ . At the end-point the solution changed from a bluish green color to a clear violet color. A 5 ml. microburette calibrated in 0.02 ml. divisions was used for the titration.

5 N sulfuric acid. 197.0 gm. of c.p.  $\text{H}_2\text{SO}_4$  (chloride-free) were dissolved in 1 liter of solution.

0.1 N potassium dichromate. 4.9037 gm. of dry c.p.  $K_2Cr_2O_7$  were dissolved in 1 liter of solution.

TABLE I

*Determination of Chlorides in Folin-Wu Blood Filtrates*

2 ml. samples, equivalent to 0.2 ml. of whole blood, were used.

	NaCl found	
	Diphenylamine indicator	Volhard method
	mg. per cent	mg. per cent
Sample 1	386.0	390.0
	392.0	390.0
	392.0	394.0
	386.0	394.0
Average.....	389.0	392.0
Sample 2	422.0	424.0
	422.0	435.0
	420.0	415.0
Average.....	421.0	425.0

TABLE II

*Determination of Chlorides in Urine (0.8 Ml.)*

NaCl found		
	Diphenylamine indicator	Volhard method
	<i>mg. per cent</i>	<i>mg. per cent</i>
Sample 1	1340.0	1350.0
	1340.0	1340.0
	1335.0	1330.0
Average.....	1338.0	1340.0
Sample 2	1006.0	990.0
	1006.0	1010.0
	995.0	1010.0
Average.....	1003.0	1003.0

Diphenylamine solution. 0.20 gm. of diphenylamine was dissolved in 100 ml. of concentrated c.p.  $H_2SO_4$  (chloride-free).

Blood filtrates. 2.0 ml. of Folin-Wu blood filtrate were

pipetted into a test-tube of 30 ml. capacity; the sides of the tube were washed with 2.0 ml. of 5 N  $\text{H}_2\text{SO}_4$ , and 4 drops of 0.2 per cent diphenylamine in concentrated  $\text{H}_2\text{SO}_4$  and 0.2 ml. of 0.1 N  $\text{K}_2\text{Cr}_2\text{O}_7$  were added. The blue color was allowed to develop by standing for a minute; then 5 drops of caprylic alcohol were added and the solution titrated with the 0.02 N silver nitrate. The silver chloride formed gives a green color in the presence of the indicator. At the end-point, when the solution is well shaken, flocculation takes place and at the same time the solution turns clear and violet. The results of a series of determinations on blood filtrates as checked against the Volhard method are shown in Table I.

Urine. 0.2 ml. of urine was added to a test-tube of 30 ml. capacity. The sides of the tube were washed with 4.0 ml. of 5 N  $\text{H}_2\text{SO}_4$ , and 6 drops of the 0.2 per cent diphenylamine solution and 0.3 ml. of 0.1 N  $\text{K}_2\text{Cr}_2\text{O}_7$  were added. The color was allowed to develop for a minute; then about 7 drops of caprylic alcohol were added and the solution was titrated with 0.02 N silver nitrate until the violet end-point was reached. The results of a series of determinations on urine samples as checked against the Volhard method are shown in Table II.

#### DISCUSSION

Quantities in the range of 1 mg. of sodium chloride can be determined by direct titration in urine and blood filtrate with a maximum deviation of 2 per cent. The method, as outlined, is capable of a high degree of accuracy and reproducibility. The diphenylamine blue indicator gives a sharp end-point, although the amounts of diphenylamine solution and of the potassium dichromate must be varied with the amount of sodium chloride used. The silver titration should be performed fairly rapidly, as the indicator has a tendency to fade. The fading may be partially prevented by performing the titration in diffuse light or by adding traces of cholesterol to the solution before titrating. The caprylic alcohol serves the purpose of carrying the precipitated silver chloride to the surface and in helping to clarify the solution at the end-point so that the violet color can be easily observed. The results obtained were in close agreement with those obtained by the Volhard method.

This method is adaptable for the study of chloride distribution ratios in various pathological cases.

#### SUMMARY

A rapid, accurate method for the determination of chlorides in blood filtrates and urine by direct titration with silver nitrate is described in which diphenylamine blue is used as an adsorption indicator. Quantities in the range of 1 mg. of sodium chloride can be determined with a maximum deviation of 2 per cent.

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# THE CHEMISTRY OF LIGNIN

## X. LIGNIN FROM OAT STRAW\*

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In a previous communication (1) results of an investigation were presented dealing with the chemistry of lignin from barley straw. A review of the literature was then presented. Inadvertently, reference to a paper by Marion (2) dealing with the lignin of oat and wheat straws was then omitted.

In this paper results of a study of the lignins from oat straw are presented. Three lignin fractions were isolated from oat straw. The first two fractions were isolated by successive and exhaustive extractions, first with a 2 per cent alcoholic sodium hydroxide solution at room temperature, and then by refluxing with a 4 per cent aqueous sodium hydroxide solution, according to the method previously described (1). The third fraction was isolated from the straw remaining from the extraction operations referred to above with fuming hydrochloric acid. The composition of the first lignin fraction agreed with that represented by the formula  $C_{40}H_{48}O_{16}$ . On the basis of a compound having a molecular weight represented by this formula, approximately four methoxyl groups and four hydroxyl groups were shown to be present. Of the four hydroxyl groups, two could be methylated with diazomethane, thus indicating that these are more acidic, possibly phenolic or enolic in character. On methylation with dimethyl sulfate and sodium hydroxide solution (9 per cent) incomplete methylation of the four hydroxyl groups resulted. However, by remethylating this preparation with dimethyl sulfate and 40 per cent sodium hydroxide solution, a product was obtained containing five methoxyl groups in addition to those naturally present in this lignin

\* Industrial Farm Products Research Division Contribution No. 260.



fraction, thus indicating that this drastic treatment resulted in the formation of one more hydroxyl group which was then methylated by the dimethyl sulfate.

When this lignin fraction was subjected to fusion with potassium hydroxide, protocatechuic acid (isolated as its dimethyl ether, veratric acid) in approximately 4 per cent yield was obtained.

The analytical results on the second lignin fraction are more in agreement with those represented by the formula  $C_{40}H_{42}O_{16}$ . In this lignin fraction approximately four methoxyl groups and four hydroxyl groups were shown to be present. Two of the hydroxyl groups could be methylated with diazomethane.

The methoxyl content of the third lignin fraction was found to be about the same as that of the other two lignin fractions. This lignin fraction was found to contain a higher percentage of carbon than either of the other two lignin fractions.

By means of a method previously described (3), the alkoxy groups in the three lignin fractions were shown to be methoxyls. The presence of other alkoxy groups, such as, for example, ethoxyl groups, was definitely excluded.

The three lignin fractions when distilled with 12 per cent hydrochloric acid afforded small quantities of formaldehyde. The significance of the isolation of this fission product from lignin from the standpoint of the presence of a methylene dioxide group in this substance has already been discussed in the paper referred to (1).

In comparing the lignin fractions isolated from oat straw with the corresponding lignin fractions isolated from barley straw one is led to the conclusion that, if these lignins are not identical, they are certainly closely related.

#### EXPERIMENTAL

1 kilo of ground oat straw (910 gm. of moisture-free material) was subjected to an exhaustive extraction with an alcoholic sodium hydroxide solution according to the procedure described in a previous communication (1). The crude lignin thus obtained, after being dried in a desiccator over sulfuric acid, amounted to 61 gm. (6.7 per cent yield calculated on the weight of dry straw taken). The weight of the straw which had been extracted with the alcoholic sodium hydroxide solution was 855

gm. (dry weight). The crude lignin was treated with 1 liter of an acetone-alcohol solution (2 volumes of acetone to 1 volume of 95 per cent ethanol), the lignin solution was filtered, and the alcohol and acetone were removed by distillation under reduced pressure. The lignin thus obtained was washed with water and dried at 56° over phosphorus pentoxide in the vacuum Abderhalden drier. The yield of this purified lignin amounted to 30 gm. (3.3 per cent calculated on the weight of dry straw taken). The lignin obtained was a light tan amorphous powder. A carbon and hydrogen determination made upon the lignin gave the following results: Found, C 62.6, 62.5, H 6.2, 6.1.

The lignin gave no test for furfural with aniline acetate paper when distilled with 12 per cent hydrochloric acid.

The nature of the alkoxyl group present in this lignin fraction was established by the method of Willstätter and Utzinger (4). The crystalline trimethylphenylammonium iodide was identified by its melting point and mixed melting point. In order to prove definitely that no alkoxyl groups other than methoxyl are present in this lignin fraction, the method described by one of us in a previous communication (3) was used. Found, (Zeisel method)  $\text{OCH}_3$  14.76, 15.04, (Kirpal and Bühn method) 14.84, 14.88. The ratio of the percentage of total carbon to the percentage of carbon present in the form of methoxyl is 10.8:1.

The data obtained on the percentages of carbon and hydrogen in this lignin fraction agree with the percentage composition as represented by a compound of the empirical formula  $\text{C}_{40}\text{H}_{48}\text{O}_{18}$ . The calculated percentages are C 62.50, H 6.25. The percentage of methoxyl found in this lignin fraction is somewhat low as compared with that calculated for four methoxyl groups in a compound represented by the above formula. The results obtained correspond to 3.7 methoxyl groups in every unit represented by the above formula.

*Distillation with 12 Per Cent Hydrochloric Acid*—2 gm. of lignin were distilled with 12 per cent hydrochloric acid by the procedure recommended by the Association of Official Agricultural Chemists for the determination of pentosans. The distillate was neutralized with sodium bicarbonate, then made slightly acid with acetic acid, and redistilled, and the first 50 cc. of distillate were retained. This distillate was treated with an alcoholic

solution of dimethylcyclohexanedione, as suggested by Weinberger (5). The crystalline precipitate was recrystallized from dilute alcohol, m.p. 191–192°, mixed m.p. 191–192°. The presence of formaldehyde in the distillate was, therefore, definitely established.

*Alkali Fusion*—To 50 gm. of potassium hydroxide contained in a nickel crucible, 25 cc. of water and 10 gm. of zinc dust were added and the mixture was heated to 100°. To these were added portionwise 5 gm. of lignin while the reaction mixture was stirred. The temperature of the mixture was gradually raised to 250° and maintained at that temperature for 30 minutes. The reaction was completed by heating the mixture at 310° for 15 minutes. The melt was allowed to cool and then dissolved in water, filtered, acidified with dilute sulfuric acid, and distilled in a current of steam until the distillate no longer gave an acid reaction. The total distillate was titrated with 0.1 N sodium hydroxide solution, phenolphthalein being used as the indicator. 181 cc. of the alkali were required. The entire neutralized distillate was evaporated to dryness on the steam bath. The acid *p*-toluide was prepared according to the method of Mulliken (6). The toluide melted at 148–149°, and, when mixed with some pure acet-*p*-toluide, there was no depression in the melting point. The acid was, therefore, acetic acid.

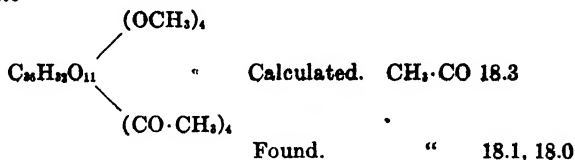
The solution remaining in the distilling flask was filtered and the filtrate was repeatedly extracted with ether. After removal of the ether a dark brown syrup was obtained. To this were added 25 cc. of 10 per cent sodium hydroxide solution, followed by 10 cc. of dimethyl sulfate. The methylation was completed by warming the reaction mixture on the steam bath. The cooled solution was extracted with ether and after removal of the ether a small quantity of syrup was obtained which had the odor of veratrole. The amount obtained was insufficient for identification.

The alkaline solution which had been extracted with ether was acidified with hydrochloric acid and again extracted with ether. After removal of the ether a gummy residue was obtained. This was boiled with water, decolorized with norit, filtered, and concentrated. Crystals were obtained which, after three recrystallizations, melted sharply at 180–181° (corrected). When mixed with some pure veratric acid, no depression in the melting point

was observed. The yield of pure material amounted to 0.2 gm. = 4 per cent of the weight of lignin.

**Acetylation**—The lignin was acetylated by the method described in a previous investigation (7). The product, light gray in color, was dried at 56° in the Abderhalden drier over phosphorus pentoxide.

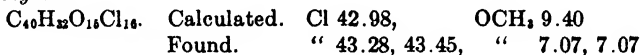
*Analysis*—



The percentage of acetyl was determined by the method described by one of us (8).

**Chlorination**—The chlorination of this lignin fraction was carried out as described in a previous communication (7). The product was colored lemon-yellow.

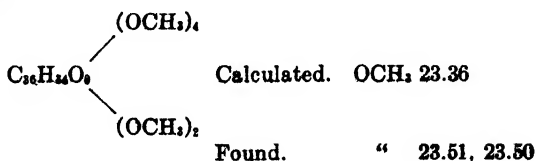
*Analysis*—



It will be noted that there was a loss of methoxyl brought about by the chlorination. A similar observation has been made in connection with the bromination of lignin from spruce wood (9).

**Methylation with Diazomethane**—2 gm. of lignin were added to an ether solution containing approximately 2 gm. of diazomethane and the mixture allowed to stand at room temperature with occasional stirring for 5 days. The product was filtered off, air-dried, and finally dried *in vacuo* at 56° over phosphorus pentoxide. A light tan amorphous product was obtained.

*Analysis*—

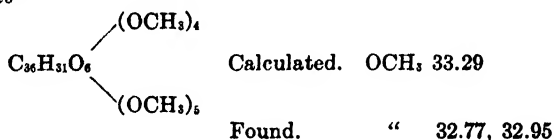


The methylation with diazomethane was repeated but no increase in the methoxyl content was obtained.

*Methylation with Dimethyl Sulfate*—3 gm. of lignin were dissolved in a solution containing 10 gm. of sodium hydroxide in 100 cc. of water, and 24 cc. of dimethyl sulfate were added drop by drop, and the reaction mixture was stirred mechanically. After all the dimethyl sulfate had been added, the reaction mixture was heated on the steam bath for 30 minutes. The product was filtered off, washed with water, and dried over phosphorus pentoxide. A methoxyl determination gave the following results: Found,  $\text{OCH}_3$  28.37, 28.40.

The above product was remethylated with dimethyl sulfate and 40 per cent hydroxide solution. A vigorous reaction took place, and, after it had subsided, the reaction mixture was heated on the steam bath for 20 minutes. The product was washed with water and dried in the Abderhalden drier at  $56^\circ$  over phosphorus pentoxide.

*Analysis*—



*Isolation of Second Lignin Fraction*—The straw (855 gm.) which had been extracted exhaustively with alcoholic sodium hydroxide solution, was treated with a sufficient 4 per cent aqueous sodium hydroxide solution to cover it completely and heated under the reflux condenser at  $100^\circ$  for 4 hours. The reaction mixture was filtered and the lignin precipitated by the addition of concentrated hydrochloric acid to the filtrate. The residual straw was again treated with a 4 per cent sodium hydroxide solution, and the operation was repeated until the alkaline extract when acidulated with hydrochloric acid no longer gave a precipitate of lignin. The cellulosic residue obtained amounted to 448 gm. It still contained 4.2 per cent of lignin as determined later by the fuming hydrochloric acid method. The lignin obtained from the several extractions was combined and purified by dissolving it in 500 cc. of 2 per cent aqueous sodium hydroxide solution and adding to it 1 liter of 95 per cent ethanol. The precipitate was filtered off, the filtrate was acidified with hydrochloric acid, and the alcohol was removed by distillation. The lignin was washed with water until the wash water was free of chlorides and dried *in vacuo* at  $56^\circ$

over phosphorus pentoxide. Yield, 9 gm. An amorphous brown substance was obtained.

*Analysis—*

Found. C 61.8, 61.8, H 5.3, 5.4, OCH<sub>3</sub> 14.68, 14.84 (ash-free basis)

The alkoxyl groups present in this lignin fraction were identified as methoxyls. The method previously referred to for the identification of the alkoxyl groups was used.

The percentage composition of a compound represented by the formula C<sub>40</sub>H<sub>48</sub>O<sub>16</sub> is C 61.68, H 5.43. The calculated percentage of methoxyl in a compound of this empirical formula having three methoxyl groups is 11.95 per cent, and on the basis of four methoxyl groups it is 15.92 per cent. The lignin fraction consists chiefly of a substance containing four methoxyl groups admixed with some material containing fewer methoxyl groups.

*Acetylation*—This was carried out according to the method previously referred to. The percentage of acetyl was determined by the same method used for the analysis of the acetyl derivative of the first lignin fraction.

*Analysis—*

C<sub>40</sub>H<sub>48</sub>O<sub>16</sub>(CO·CH<sub>3</sub>)<sub>4</sub>. Calculated. CH<sub>3</sub>·CO 18.1  
Found. " 16.68, 16.39

*Methylation with Diazomethane*—0.5 gm. of lignin was added to an ether solution containing approximately 1.8 gm. of diazomethane and allowed to remain at room temperature for 5 days. The product was filtered off and dried *in vacuo* at 56° over phosphorus pentoxide.

*Analysis—*

$$\begin{array}{c} \text{(OCH}_3\text{)}_4 \\ \diagup \\ \text{C}_{40}\text{H}_{48}\text{O}_{16} - \text{(OH)}_2 \\ \diagdown \\ \text{(OCH}_3\text{)}_2 \end{array}$$
 Calculated. OCH<sub>3</sub> 23.0  
Found. " 22.32, 22.50

*Distillation with 12 Per Cent Hydrochloric Acid*—The distillation of the lignin with the subsequent cohobation of the distillate was carried out as previously described. Formaldehyde was identified in the distillate by the dimethylcyclohexanedione method already referred to.

*Isolation of the Residual Lignin*—A portion of the residual straw

(112 gm. = 25 per cent of the weight of the straw) which had been ground fine enough to pass through an 80-mesh sieve was boiled for 3 hours under a reflux condenser with 5 liters of distilled water to which sufficient hydrochloric acid had been added to neutralize the sodium hydroxide adhering to the straw. The straw was filtered off and boiled again for 3 hours under a reflux condenser with 5 liters of 2 per cent hydrochloric acid solution, again filtered off, washed with distilled water until free of acid, and dried at 105°. Yield, 73.5 gm. = 65.6 per cent of the weight of the straw. The dry and extracted straw was added portionwise to 1000 cc. of fuming hydrochloric acid ( $d$  1.212 to 1.223 at 15°) which was maintained at a temperature of +8° to +10°. A stream of dry hydrochloric acid gas was passed through the reaction mixture for 2 hours. It was then allowed to stand in an ice box (+8° to +10°) for 24 hours. The reaction mixture was diluted with 10 liters of distilled water and boiled under a reflux condenser for 1 hour. It was allowed to cool to room temperature and was then filtered off and washed with distilled water until free of acid. It was dried at 105° and a brown-colored product was obtained. Yield, 4.8 gm. = 4.2 per cent of the original (112 gm.) unextracted straw.

*Analysis*—(Ash-free basis)

Found. C 64.7, 64.7, H 5.8, 5.8, OCH<sub>3</sub> 14.96, 14.86

The character of the alkoxyl group present in this lignin fraction was established by the methods previously described.

When this lignin fraction was distilled with 12 per cent hydrochloric acid, some formaldehyde was obtained in the distillate.

#### SUMMARY

1. Three lignin fractions were isolated from oat straw by extracting it successively and exhaustively, first with a 2 per cent alcoholic sodium hydroxide solution at room temperature, then by refluxing it with a 4 per cent aqueous sodium hydroxide solution, and finally subjecting the residue to the action of fuming hydrochloric acid. The composition of the first lignin fraction agreed with that represented by the formula  $C_{40}H_{48}O_{16}$ . Four methoxyl groups and four hydroxyl groups were found to be present. Of the four hydroxyl groups, two could be methylated with diazomethane, thus indicating that these are more acidic, possibly

phenolic or enolic in character. When fused with potassium hydroxide, protocatechuic acid was obtained. The yield was approximately 4 per cent of the weight of the lignin. The analytical results on the second lignin fraction are more in agreement with that represented by the formula  $C_{40}H_{42}O_{16}$ . In this lignin fraction, approximately four methoxyl groups and four hydroxyl groups were shown to be present. Two of the hydroxyl groups could be methylated with diazomethane. The third lignin fraction was found to have a higher percentage of carbon than either of the other two lignin fractions. The percentage of methoxyl did not differ from that of the other two fractions. .

2. The alkoxyl groups present in all the three lignin fractions were proved definitely to be methoxyls.

3. All the three fractions when distilled with 12 per cent hydrochloric acid afforded some formaldehyde.

4. The lignin fractions isolated from oat straw are closely related to the corresponding lignin fractions isolated from barley straw.

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## ALLOCHOLESTEROL AND EPIALLOCHOLESTEROL\*

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The reduction of  $\alpha,\beta$ -unsaturated ketones to the corresponding unsaturated alcohols by the use of aluminum alcoholates was first described by Meerwein and Schmidt (3). The reaction has been successfully applied to sterol ketones by several investigators (4, 5). We have subjected cholestenone (I), the unsaturated ketone corresponding to allocholesterol, to the action of aluminum isopropylate and find that the carbonyl group is reduced, leaving the double bond intact. Since the reduction of the keto group introduces a new asymmetric carbon atom, the reaction should lead to a mixture of two epimeric unsaturated alcohols. These alcohols should correspond to allocholesterol rather than cholesterol, since the double bond in cholestenone is at position 4-5 (6).

The principal product of the reduction of cholestenone is, in fact, an addition compound of two isomeric sterols. This molecular compound has the composition  $C_{27}H_{46}O$  and can be repeatedly recrystallized without alteration of its physical properties (m.p.  $141^\circ$ ;  $[\alpha]_D^{22} = +83.9^\circ$ ). The compound can be resolved into its components by digitonin, since only one of the constituent sterols forms an insoluble digitonide. The precipitation with digitonin indicates that the configuration of the hydroxyl group in this sterol

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The preparation of epiallocholesterol has been described in a preliminary communication (1). A portion of this work was also reported at the meeting of the American Society of Biological Chemists at Washington, April, 1936 (2).

† Columbia University Fellow, 1934-36. This report is from a dissertation submitted by E. A. Evans, Jr., in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

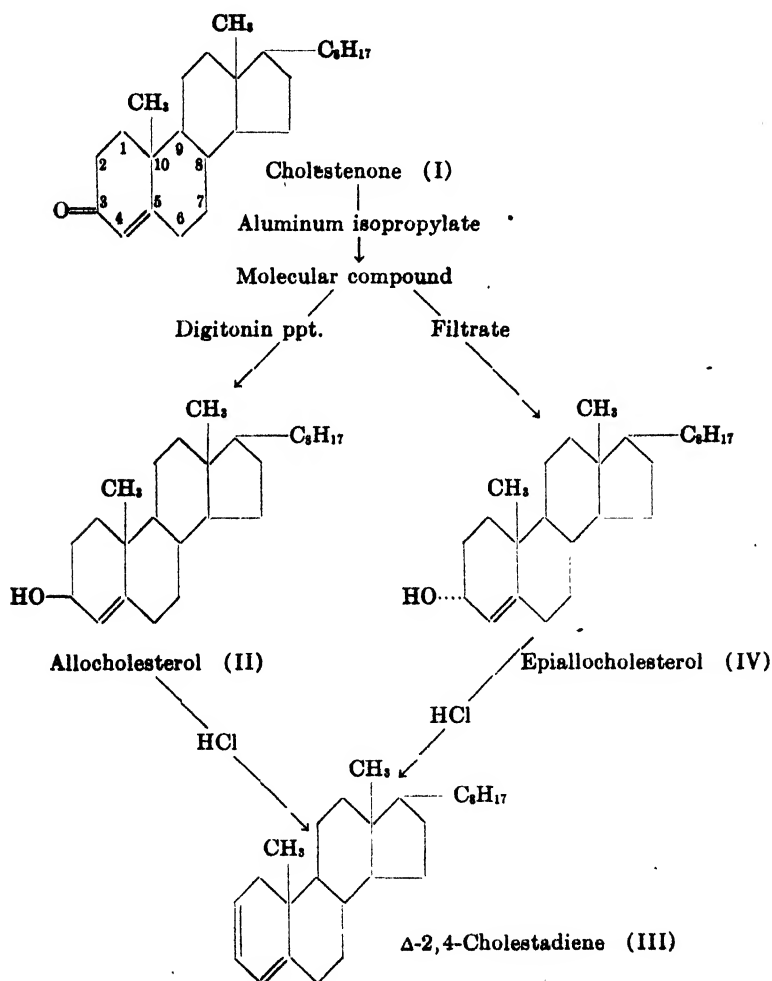
corresponds to that of cholesterol and the other naturally occurring sterols (7). After liberation from the digitonide by treatment with pyridine and ether in the cold (8), this sterol proved to be an unsaturated alcohol, isomeric with cholesterol, melting at  $132^{\circ}$ ,  $[\alpha]_D^{25} = +43.7^{\circ}$ . It gives an intense red color with trichloroacetic acid in the cold (Rosenheim reaction) and forms a crystalline acetate, melting at  $85^{\circ}$ .

This compound should, from its method of preparation, be allocholesterol. Its properties, however, differ markedly from those reported for allocholesterol prepared by the elimination of HCl from cholesterol hydrochloride (9-12). The product of the latter reaction melts at  $117^{\circ}$  (9) or  $121-122^{\circ}$  (11), has a slight positive rotation ( $0-8^{\circ}$ ), gives a less intense Rosenheim reaction than our sterol, and on catalytic hydrogenation yields a mixture of coprosterol and dihydrocholesterol. Our sterol is converted on hydrogenation into coprosterol; dihydrocholesterol could not be isolated, though its presence in small amounts cannot be excluded.

The most striking property of the allocholesterol of the literature was reported to be the ease of its rearrangement to cholesterol, especially on heating or in the presence of acids (9-13). When our sterol is refluxed with dilute alcoholic HCl, not only is there no formation of cholesterol, but a complete loss of digitonin precipitability occurs. Under these conditions the unsaturated sterol loses 1 mole of water with the formation, in quantitative yield, of a double unsaturated hydrocarbon melting at  $79^{\circ}$ ,  $[\alpha]_D^{25} = -112.5^{\circ}$ . The properties of this compound are similar to those of cholesterolene, which is formed by the dehydration of cholesterol or by the elimination of HCl from cholesteryl chloride. The ultra-violet absorption of our hydrocarbon differs from that of cholesterolene (14), however, in showing maxima at much shorter wave-lengths (229, 235, and  $240\ m\mu$ ). By analogy to the general behavior of  $\alpha,\beta$ -unsaturated alcohols the ease of dehydration and formation of a conjugated bond system speaks for the presence of the double bond at position 4-5 in the alcohol. The hydrocarbon, therefore, is the previously undescribed  $\Delta$ -2,4-cholestadiene (III).

The precipitation by digitonin, the reduction to coprosterol, and the ease of dehydration are difficult to reconcile with any formula for the new sterol other than that assigned to allocholesterol. In order to determine the source of the discrepancy be-

tween the properties of the new sterol and those of the known allocholesterol, a sample of allocholesterol prepared by Windaus' procedure (9) was treated with dilute alcoholic HCl. Under



these conditions there was a 40 to 60 per cent loss of digitonin precipitability. The material still precipitating with digitonin after the acid treatment was cholesterol, while the mother liquor

contained the same hydrocarbon obtained from our sterol in quantitative yield. A synthetic mixture of 60 per cent cholesterol with 40 per cent of the new sterol was similar, in melting point and rotation, to the allocholesterol of the literature.

We conclude from this evidence that the new sterol is pure allocholesterol (II), and that the substance previously assigned this name is a mixture of cholesterol and allocholesterol. We have not succeeded in rearranging our allocholesterol to cholesterol, experiments to this end usually leading to varying yields of  $\Delta$ -2,4-cholestadiene. It is apparent, therefore, that the generally accepted belief in the ready transformation of allocholesterol to cholesterol is erroneous. The fact that cholesterol was so easily obtained from "allocholesterol" can be explained by the dehydration of the allocholesterol under the conditions adopted, whereby the isolation of the cholesterol already present was facilitated.

The second product of the reduction of cholestenone proved to be an alcohol,  $C_{27}H_{46}O$ , not precipitable by digitonin, melting at  $84^\circ$ ,  $[\alpha]_D^{24} = +120.8^\circ$ . This compound likewise gives an intense Rosenheim reaction and is also, on treatment with dilute alcoholic HCl, dehydrated to  $\Delta$ -2,4-cholestadiene. On catalytic hydrogenation the sterol forms a mixture of epicoprosterol and epidihydrocholesterol but no digitonin-precipitable compound. The unsaturated sterol is, therefore, the hitherto unknown epiallocholesterol (IV).<sup>1</sup>

In all of the known sterols and bile acids, the steric relation between Rings B, C, and D is the same, corresponding most probably to that of *trans*-decalin. Isomerism is only known to occur at carbon atom 5 (shared by Rings A and B) and with the hydroxyl at carbon atom 3. The hydrogen atom at carbon atom 5 may be either *cis* or *trans* to the methyl group at carbon atom 10, giving rise respectively to compounds of the coprostane type (*cis*-decalin) and the cholestane type (*trans*-decalin). Similarly, two known groups of isomers originate from the two possible configurations of the hydroxyl at carbon atom 3 (16, 17).

Following the proposal of Ruzicka, the configuration of substituents at carbon atom 3 is designated by the prefix *cis* or *trans*, the configuration being expressed in terms of its relationship to the

<sup>1</sup> Marker, Oakwood, and Crooks have recently reported the preparation of epicholesterol (15).

hydrogen at carbon atom 5. A second prefix is added to indicate the type of fusion between Rings A and B (*cis*- or *trans*-decalin). Dihydrocholesterol is accordingly designated *trans-trans*; epico-prosterol as *trans-cis*. While this nomenclature can be satisfactorily applied to the saturated sterols, difficulties are encountered with the unsaturated sterols in which the hydrogen at carbon atom 5 is absent. The unsaturated hormone, dehydroandrosterone (18), was designated *trans*, since on hydrogenation a derivative corresponding in configuration to dihydrocholesterol is obtained. The nomenclature is, however, inapplicable to the new unsaturated sterols, epicholesterol and epiallocholesterol. Since cholesterol, on hydrogenation, yields dihydrocholesterol, the hydroxyl group may, on this basis, be designated *trans*. Allocholesterol, to the contrary, is hydrogenated to coprosterol and the hydroxyl group in this compound would carry the same prefix as that in epicholesterol, namely *cis*. Epiallocholesterol and cholesterol would likewise carry the same prefix, *trans*, although the hydroxyl groups in the two compounds have opposite configurations.

It seems to us, therefore, highly advantageous to describe the configuration of the hydroxyl group in terms of its steric relationship to the methyl group at carbon atom 10. The stereochemical relation of this methyl group to the rest of the molecule is the same in all known sterols and bile acids, while the hydrogen at carbon atom 5 can have either of two configurations or be absent entirely. The choice of the methyl group at carbon atom 10 as a fixed point of reference in assigning spatial configurations to other groups gives a system of nomenclature applicable to unsaturated as well as saturated sterols. This is demonstrated in Table I.

According to this nomenclature all those sterols in which the hydroxyl has a configuration corresponding to that in cholesterol, and are therefore precipitable with digitonin, carry the same prefix, *cis*. All epi forms are designated *trans* and these compounds do not precipitate with digitonin. This system of nomenclature also permits a direct expression of the findings of Lettré (19), who has shown that molecular compound formation occurs only between those saturated sterols which differ at both carbon atoms 3 and 5 in their stereochemical configuration. Dihydrocholesterol (*cis-trans*) forms a molecular compound, for

example with epicoprosterol (*trans-cis*), as do likewise epidihydrocholesterol (*trans-trans*) and epicoprosterol (*cis-cis*). These relationships are not expressed in the old nomenclature: dihydrocholesterol (*trans-trans*) and epicoprosterol (*cis-trans*).

All of the new compounds, allocholesterol, epiallocholesterol, and the  $\Delta$ -2,4-cholestadiene formed by their dehydration, give immediate and intense red colors with 90 per cent trichloroacetic acid (Rosenheim reaction). This color test was regarded by Rosenheim (20) as characteristic of those sterols possessing a double bond at position 4-5. We have subjected a number of sterols of known constitution to this reaction and find that only those compounds give a positive test in which either a conjugated

TABLE I

*Position of OH Group at Carbon Atom 3 and of the H Atom at Carbon Atom 5 Relative to CH<sub>3</sub> Group at Carbon Atom 10 in Sterols*

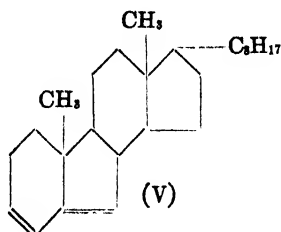
	OH group	H atom	Precipitability with digitonin
Dihydrocholesterol.....	<i>Cis</i>	<i>Trans</i>	+
Epidihydrocholesterol.....	<i>Trans</i>	"	-
Coprosterol.....	<i>Cis</i>	<i>Cis</i>	+
Epicoprosterol.....	<i>Trans</i>	"	-
Cholesterol.....	<i>Cis</i>		+
Epicholesterol.....	<i>Trans</i>		-
Allocholesterol.....	<i>Cis</i>		+
Epiallocholesterol.....	<i>Trans</i>		-

system of double bonds is present, or in which a double bond is in the  $\alpha,\beta$  position to a hydroxyl group. The ease with which allocholesterol and epiallocholesterol are dehydrated makes it very probable that a conjugated double bond system is the basic factor in the color reaction, since the trichloroacetic acid can, in all probability, duplicate the action of the dilute hydrochloric acid. The Rosenheim reaction, therefore, is given by those sterols possessing a conjugated double bond system or by those capable of forming such a system by the action of the reagent. The reaction is negative both for  $\alpha,\beta$ -unsaturated ketones (cholestenone, 7-oxocholesterol), even when the carbonyl group is adjacent to a conjugated double bond system (oxycholesterilene) (see Table II). The carbonyl group must therefore interfere with the reaction.





The hydrocarbon  $C_{36}H_{48}$  described by Windaus and Resau (34) and by Lettré (35), which contains a conjugated double bond system (V), also gives a positive reaction.<sup>2</sup>



#### EXPERIMENTAL

*Reduction of Cholestenone with Aluminum Isopropylate*—21 gm. of cholestenone were dissolved in 200 cc. of absolute isopropyl alcohol. To this was added a solution of 20 gm. of aluminum isopropylate, prepared according to Adkins (36), in 200 cc. of absolute isopropyl alcohol. The solution was refluxed at such a rate that slow distillation occurred, the reaction being continued until the Legal test for acetone in the distillate was almost negative (10 hours). The solution was cooled in ice, and 200 cc. of ether and a sufficient quantity of cold, 20 per cent KOH were added to produce two clear layers. The aqueous layer was removed, and the ether was washed repeatedly with water and dried over  $Na_2SO_4$  and  $K_2CO_3$ . The ether was removed at room temperature in an atmosphere of  $CO_2$ . The crystalline residue was dissolved in 150 cc. of dry, boiling ether, an equal volume of acid-free methyl alcohol added, and the solution slowly cooled, finally in an ice-salt mixture. The crystalline material was filtered off and dried. Yield, 10.9 gm.; m.p.  $141^\circ$ .<sup>3</sup>

For analysis the material was dried for 12 hours *in vacuo* over  $P_2O_5$  at  $60^\circ$ .

$C_{27}H_{46}O$ .	Calculated.	C 83.86, H 12.00
	Found.	" 84.15, " 12.07
$[\alpha]_D^{25}$	$= +84.1^\circ$ (2 per cent in benzene)	

<sup>2</sup> We are indebted to Miss Sarah Ratner for a sample of this substance as well as for Compounds 24 and 25 in Table II.

<sup>3</sup> All melting points are corrected.

*Isolation of Allocholesterol*—6 gm. of the reduction product were dissolved in 600 cc. of absolute ethyl alcohol and treated with 11 gm. of digitonin in 750 cc. of 95 per cent ethyl alcohol, the precipitate being filtered off after 24 hours. The dry digitonides (11.3 gm.), after extraction in a Soxhlet apparatus with dry ether for 1 hour, were dissolved in 200 cc. of cold, dry pyridine and 300 cc. of dry ether were added. The precipitate was filtered off and washed with large volumes of ether. The filtrate was repeatedly extracted with large volumes of water to remove the pyridine. The ether solution was dried over  $\text{Na}_2\text{SO}_4$  and  $\text{K}_2\text{CO}_3$  and the ether removed in the cold by a stream of dry  $\text{CO}_2$ . The crystalline residue was purified by dissolving in ether at room temperature, adding an equal volume of methyl alcohol, and removing the ether by a stream of  $\text{CO}_2$  until crystallization began. By cooling the mixture in an ice-salt bath, the sterol came out as long needles, melting at  $132^\circ$ ;  $[\alpha]_D^{23} = +43.7^\circ$  (1 per cent in benzene). On analysis, even after drying in a high vacuum (which must be done at room temperature), the substance gave slightly low values for carbon and hydrogen, although no change in melting point or rotation was observed after repeated recrystallization from various media, indicating that the substance retains solvents.<sup>4</sup> The average of four analyses of different preparations gave C 83.28, H 11.45; calculated for  $\text{C}_{27}\text{H}_{46}\text{O}$ , C 83.86, H 12.00.

The compound is easily soluble in benzene, acetone, ether, chloroform, dioxane, and pyridine; less so in methyl and ethyl alcohols. It gives positive Rosenheim, Salkowski, and Liebermann-Burchard reactions.

*Allocholesterol Acetate*—50 mg. of allocholesterol were dissolved in 0.5 cc. of dry pyridine and 1 cc. of acetic anhydride was added. The solution stood at room temperature for 18 hours. The acetate was precipitated by ice water and twice recrystallized from dilute methyl alcohol. Long white needles, melting at  $85^\circ$ , were obtained.

For analysis the material was dried over  $\text{P}_2\text{O}_5$  at room temperature *in vacuo*.

$\text{C}_{27}\text{H}_{46}\text{O}_2$ .	Calculated.	C 81.26, H 11.12
	Found.	" 81.24, " 11.11

<sup>4</sup> A similar difficulty is frequently encountered with sterols and sterol derivatives (4). The excellent analytical values of the allocholesterol acetate indicate that the alcohol is homogeneous.

*Preparation of  $\Delta$ -2,4-Cholestadiene from Allocholesterol*—100 mg. of allocholesterol were dissolved in 15 cc. of 95 per cent ethyl alcohol, 2 drops of concentrated HCl added (solution approximately N/30 in HCl), and the solution refluxed for 2 hours. A large volume of water was added and the precipitate was extracted with ether. The ether was removed and the residue, which gave no precipitate with digitonin, was recrystallized from acetone. The yield was quantitative. Long needles, melting at 79°, were obtained. The hydrocarbon is very soluble in ether, petroleum ether, and benzene; considerably less so in ethyl and methyl alcohols. It gives positive Rosenheim and Salkowski reactions. For analysis the hydrocarbon was dried *in vacuo* over  $P_2O_5$  at room temperature.

$C_{27}H_{44}$ . Calculated. C 87.96, H 12.04  
Found. " 87.67, " 12.14  
 $[\alpha]_D^{25} = -112.5^\circ$  (2 per cent in benzene)

Perbenzoic acid titration gave 1.39 and 1.49 moles after 24 hours; 1.56 and 1.66 moles after 48 hours; 1.63 and 1.74 moles after 82 hours.

The absorption spectrum of the hydrocarbon is given in Fig. 1.

*Reduction of Allocholesterol*—458 mg. of allocholesterol were dissolved in 25 cc. of amyl ether and shaken in hydrogen at atmospheric pressure and room temperature with 300 mg. of platinum oxide for 120 hours. It was not possible to obtain complete hydrogenation, the Rosenheim and Salkowski reactions still being slightly positive after prolonged shaking. The unsaturated sterols were accordingly removed by the method of Schoenheimer (37). The crystalline residue was dissolved in 25 cc. of 95 per cent ethyl alcohol; 1 per cent bromine in ethyl alcohol was added slowly over a period of 2 hours with ice cooling until a slight permanent color indicated the presence of a slight excess of bromine; 1.5 gm. of digitonin in ethyl alcohol were then added and the solution let stand for 24 hours. The precipitate was filtered off, washed with alcohol, and extracted for 1 hour in the Soxhlet apparatus with dry ether. The digitonide was decomposed by dissolving in dry pyridine, dry ether being added until no further precipitation occurred, and the digitonin filtered off. The filtrate was washed with dilute acid to remove the pyridine and the

ether evaporated. Rosenheim and Salkowski reactions were now negative. As we were unsuccessful in fractionating pure coprosterol or dihydrocholesterol from the product, we applied the method of Windaus and Uibrig (33, 38) in which the sterols are partially converted into their epimers by the action of sodium in xylene. The material was accordingly dissolved in 25 cc. of dry xylene, 0.5 gm. of sodium added, and the solution refluxed for 8 hours. The sodium was converted to ethoxide by addition of alcohol, ether added, and the ether washed free of alkali by re-

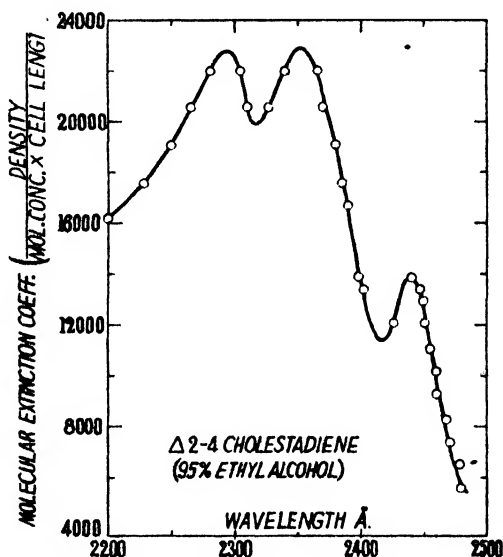


FIG. 1. Absorption spectrum of  $\Delta^2,4$ -cholestadiene

peated extraction with water. After removal of the ether, the residue was taken up in 25 cc. of 95 per cent ethyl alcohol and a solution of 0.5 gm. of digitonin in 100 cc. of ethyl alcohol was added. The digitonides were filtered off after 24 hours. The filtrate and washings were taken to dryness *in vacuo* and the residue repeatedly extracted with dry ether. The ether was removed at room temperature and the residue repeatedly crystallized from methyl alcohol and acetone. Epicoprosterol was obtained; m.p. 116–117°. A mixed melting point with epicoprosterol showed no depression. For further identification the

epicoprosterol was converted into its acetate; m.p.  $86^{\circ}$  (39). The epicoprosterol was unquestionably derived from coprosterol by the action of sodium and xylene, since the original reduction mixture was entirely precipitable with digitonin.

The 112 mg. of digitonide obtained by the final precipitation gave coprosterol; m.p.  $98-99^{\circ}$ . The material gave no depression of the melting point with coprosterol.

*Acid Treatment of Allocholesterol from Cholesterol Hydrochloride*—A solution of 500 mg. of allocholesterol (m.p.  $117-118^{\circ}$ ;  $[\alpha]_D^{21} = +5.3^{\circ}$ ), prepared by the standard method (9), in 15 cc. of 95 per cent ethyl alcohol was refluxed for 2 hours after the addition of 2 drops of concentrated HCl. An excess of water was added, the sterol extracted with ether, and the ether washed free of acid with water. After removal of the ether 22.9 mg. and 21.0 mg. samples of the residue gave 50.4 mg. and 47.1 mg. of digitonide respectively; i.e., 55.5 and 56.2 per cent of the residue were precipitable. One crystallization of the balance of the residue from dilute ethyl alcohol gave 239 mg. of slightly impure hydrocarbon; m.p.  $75-76^{\circ}$ . After two recrystallizations from ethyl alcohol the melting point rose to  $79^{\circ}$ ,  $[\alpha]_D^{24} = -108.2^{\circ}$ ; no depression of the melting point was observed on mixing the substance with the hydrocarbon obtained from epiallocholesterol or allocholesterol. The mother liquors were united, the solvent removed, and the material crystallized once from petroleum ether and twice from acetone. Pure cholesterol was obtained; the melting point,  $147.5^{\circ}$ , was not depressed on admixture with an authentic specimen. For further identification the cholesterol was converted to its acetate; m.p.  $113^{\circ}$ .

The separation of the hydrocarbon and cholesterol from the acid-treated material can also be carried out with digitonin, the cholesterol being thus precipitated and the hydrocarbon obtained from the filtrate. The yields of cholesterol and hydrocarbon indicate the absence of other reaction products.

*Isolation of Epiallocholesterol*—The filtrates from the digitonin precipitation in the allocholesterol preparation were taken to dryness *in vacuo* at  $30-35^{\circ}$ . The residue was repeatedly extracted in the cold with dry ether and the united ether solutions taken to dryness in an atmosphere of carbon dioxide. The pale yellow oil quickly crystallized; m.p.  $81^{\circ}$ . The crystals were dissolved in 35

cc. of acetone at 35° and 2 cc. of water were added; the solution was allowed to cool slowly and was finally placed in the refrigerator. There were thus obtained 2 gm. of rosettes of long needles, melting at 84°. Further recrystallization gave no change in melting point or rotation.

The compound is easily soluble in ether, benzene, chloroform, dioxane, and pyridine; somewhat less so in ethyl and methyl alcohols. With 90 per cent trichloroacetic acid it immediately gives an intense carmine-red color. The Salkowski and Liebermann-Burchard reactions are also positive. An alcoholic solution gave no precipitate with an excess of digitonin after 72 hours.

For analysis the material was dried *in vacuo* over  $P_2O_5$  at room temperature.

$C_{17}H_{46}O$ .	Calculated.	C 83.86, H 12.00
	Found.	" 84.12, " 11.97
$[\alpha]_D^{25} = +120.8^\circ$ (2 per cent in benzene)		

*Epiallocholesterol Acetate*—To a solution of 50 mg. of epiallocholesterol in 0.5 cc. of dry pyridine 0.5 cc. of acetic anhydride was added. After 24 hours at room temperature the acetate was precipitated with water, ether added, and the ether washed free of pyridine with water. The ether was removed and the crystalline residue was twice recrystallized from methyl alcohol. Long white needles, melting at 82.5°, were obtained. The acetate also gives an intense Rosenheim reaction.

For analysis the material was dried *in vacuo* over  $P_2O_5$  at room temperature.

$C_{19}H_{40}O_2$ .	Calculated.	C 81.24, H 11.29
	Found.	" 81.27, " 10.96

*Reduction of Epiallocholesterol*—390 mg. of epiallocholesterol in 30 cc. of amyl ether were shaken with 300 mg. of platinum oxide in hydrogen at atmospheric pressure and room temperature until the Salkowski and Rosenheim reactions were negative. This required 72 hours, small amounts of fresh catalyst being added at intervals. The solution was taken to dryness *in vacuo*. The residue gave no precipitate with digitonin. By repeated fractionation from methyl alcohol about 20 mg. of pure epidihydrocholesterol were obtained; m.p. 185°. No depression of the melting

point with epidihydrocholesterol was observed. For further identification the material was converted into the acetate; m.p. 95° (11). The mixed melting point with epidihydrocholesterol acetate showed no depression.

In accordance with the experience of Windaus we did not succeed in separating epicoprosterol from the remaining epidihydrocholesterol. The material was therefore refluxed with sodium and xylene, whereby the epidihydrocholesterol is converted largely to dihydrocholesterol while the epicoprosterol remains, for the most part, unchanged (33, 38). The dihydrocholesterol and coprosterol can then be separated from the epicoprosterol by precipitation with digitonin. The crystalline residue was accordingly dissolved in 25 cc. of dry xylene, 0.5 gm. of sodium added, and the solution refluxed for 8 hours. After the addition of alcohol, the solution was washed with water until neutral and then brought to dryness. The residue was taken up in alcohol, an excess of digitonin was added, and the precipitate was filtered off after 24 hours. The filtrate was taken to dryness and extracted with ether. The ether was removed and the residue, after repeated recrystallization from methyl alcohol, yielded epicoprosterol; m.p. 115–116°. No depression of the melting point with epicoprosterol was observed.

The epicoprosterol must have been present originally, and could not have been formed from coprosterol by the action of sodium and xylene, since the original reduction product gave no precipitate with digitonin.

*Dehydration of Epiallocholesterol*—A solution of 100 mg. of epiallocholesterol in 10 cc. of 95 per cent ethyl alcohol was refluxed for 2 hours after the addition of 2 drops of concentrated HCl. On cooling, fine white needles of  $\Delta$ -2,4-cholestadiene settled out. 80 mg. yield; m. p. 79°. An additional 18 mg. were obtained from the mother liquor.

#### SUMMARY

On reduction of cholestenone with aluminum isopropylate an addition compound of two unsaturated, isomeric sterols is formed, of which one is precipitable with digitonin. This precipitable sterol is pure allocholesterol, differing markedly in its properties from the product previously assigned this name. The sterol not

precipitating with digitonin is the previously unknown epimer of allocholesterol, epiallocholesterol.

The allocholesterol described in the literature was found to be a mixture of allocholesterol and cholesterol.

Allocholesterol and epiallocholesterol on treatment with dilute hydrochloric acid are quantitatively dehydrated to  $\Delta$ -2,4-cholestadiene.

The study of a number of sterols and sterol derivatives indicates that the Rosenheim reaction with trichloroacetic acid is characteristic of compounds containing either a conjugated double bond system or a hydroxyl group adjacent to a double bond.

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## A DIRECT MICROTITRATION METHOD FOR BLOOD SUGAR

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The proposed method depends on the reduction of ferricyanide to ferrocyanide by the sugar, followed by a direct titration of the ferrocyanide with standard ceric sulfate<sup>1</sup> in the presence of setopaline C as oxidation-reduction indicator. 0.1 cc. of blood is analyzed, and its sugar content is given directly by the burette reading, in which 0.01 cc. indicates 1 mg. per cent of blood sugar.

Ferricyanide as a sugar reagent has over copper the advantages that no added materials are required to keep in solution either the ferri- or the ferrocyanide, and especially that the reduced ferrocyanide is not readily reoxidized by air. In consequence ferricyanide has been used as reagent in several newer blood sugar methods,<sup>2</sup> which differ chiefly in the technique used to measure the

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<sup>1</sup> Discussion of the use of ceric sulfate as a standard oxidizing substance in analytical chemistry may be found in the latest editions of such textbooks as those by Kolthoff and Furman (1929) and Treadwell and Hall (1935), and also in a pamphlet on ceric sulfate issued by the G. Frederick Smith Company, 867 McKinley Avenue, Columbus, Ohio. Whitmoyer (1933, 1934) has employed ceric sulfate for direct titration of ferrocyanide in analyses of pure solutions of glucose, fructose, and invert sugar. The indicator, alphazarine G, which he found satisfactory in these analyses, gives, however, no definite end-point in dilute blood filtrates such as are used in present micromethods.

<sup>2</sup> Besides the Hagedorn-Jensen method there are the colorimetric method of Folin (1928), the gasometric and timing methods of Van Slyke and Hawkins (1928) and Hawkins and Van Slyke (1929), the potentiometric method of Shaffer and Williams (1935), and the titration method of Jonescu-Matiu (1928). Jonescu-Matiu employs a direct titration of the reduced ferricyanide with permanganate, but requires 2 to 4 cc. samples of blood.

extent of reduction. The ferricyanide method which has won widest application, and almost general use on the Continent, has been that of Hagedorn and Jensen (1923), in which a measured amount of ferricyanide is used and the excess after reduction is titrated iodometrically. This method owes its deserved popularity to a combination of advantages. It requires only 0.1 cc. of blood. After the proteins are precipitated, the coagulum is washed free of sugar and the entire filtrate used for analysis, thereby eliminating measurement of aliquots. In consequence only three precise measurements are required, the 0.1 cc. sample of blood, the 2 cc. of standard ferricyanide solution, and the burette measurement of the thiosulfate used in the titration. The titration is accurate, and the end-point is sharp. More analyses can be carried through in an hour than by most other methods.

The Hagedorn-Jensen method has nevertheless several drawbacks. (1) The sugar is estimated, not by a single direct measurement, but by the difference between the amounts of ferricyanide present before and after the reduction. Titration by difference, compared with direct titration, demands two standard solutions and two quantitative measurements instead of one, and it involves a greater probable error (see discussion by Peters and Van Slyke (1932) p. 36). (2) In order to limit the error of the titration by difference, it is necessary in the Hagedorn-Jensen method to use as little ferricyanide as possible; the amount employed is therefore sufficient to measure only 385 mg. per cent of blood sugar. If more is present, as sometimes occurs, the analysis must be repeated on a smaller sample. (3) The amount of ferricyanide reduced is not exactly proportional to the sugar. The ratio of sugar to ferricyanide reduced increases as the blood sugar nears the 385 mg. limit and the ferricyanide nears exhaustion. Hence calculation of sugar from the titration figures cannot be made with a constant factor, but requires use of an empirical curve or table. (4) The Hagedorn-Jensen method gives blood sugar values which are 10 to 30 mg. per cent higher than the fermentable sugar (Hiller, Linder, and Van Slyke, 1925).

The method here described attempts to retain the advantages that have justified the popularity of Hagedorn and Jensen's, and, at the same time, to eliminate its drawbacks. The present method utilizes ferricyanide reduction under essentially the same

conditions as the Hagedorn and Jensen, but offers the following changes in analytical procedure. (1) By titration of the reduced ferrocyanide instead of the excess ferricyanide, the titration by difference is replaced by a direct titration. (2) Because only the reduced ferrocyanide is titrated, a great excess of ferricyanide can be used, so that one may determine concentrations of blood sugar up to 800 mg. per cent. (3) The use of a large excess of ferricyanide also adds the advantage that, over this wide range of blood sugar concentrations, the amount of ferrocyanide formed is accurately proportional to the sugar, permitting calculations to be made with a constant factor. As a practical convenience we use ceric sulfate solution of such concentration that 0.01 cc. is equivalent to 1 mg. per cent of blood sugar, so that the burette reading multiplied by 100 gives the blood sugar. (4) Replacement of the zinc deproteinization technique of Hagedorn and Jensen by a modification of Fujita and Iwatake's (1931) cadmium technique decreases the non-fermentable reducing material in the blood filtrates to practically zero. We have modified the deproteinization technique of Fujita and Iwatake by adding an excess of barium carbonate to the precipitated mixture of blood and cadmium hydroxide, in order to rid the filtrate of traces of dissolved cadmium. The cadmium must be removed or it would form precipitates with the reagents later added, and interfere with the titration.

We have been unable to determine small amounts of fructose with the degree of accuracy obtained in our analyses of glucose. This difference may be due to the reaction between ceric sulfate and the  $\alpha$ -keto groups of the fructose oxidation products. Fromageot and Desnuelle (1935) have shown that keto acids reduce ceric sulfate at room temperature. We have observed that fructose reduces ceric sulfate at room temperature in acid solution much more rapidly than glucose. Presumably, enough of the reducing groups remain after oxidation of fructose with ferricyanide to interfere with the final titration by the dilute ceric sulfate. This effect is not found in the determination of glucose by our method. Other sugars than these two have not been studied.

#### *Reagents—*

(a) Alkaline ferricyanide solution. 5.00 gm. of  $K_3Fe(CN)_6$  and 10.6 gm. of anhydrous  $Na_2CO_3$  are dissolved in water and made up

to 1 liter. Reagent grade ferricyanide should be recrystallized from water, and then rendered free of ferrocyanide by the method of Folin (1928). This method is also described in Peters and Van Slyke ((1932) pp. 462-463). This solution when protected from light and dust keeps for several months.

(b) 18 N sulfuric acid (approximate). Made by adding 465 cc. of concentrated sulfuric acid (sp. gr. 1.84) to 535 cc. of water. Reagent grade sulfuric acid is used and must first be tested for reducing material as follows: To 20 cc. of the acid + 60 cc. of  $H_2O$  add 0.05 cc. of approximately 0.1 N  $KMnO_4$ . The pink color must persist for at least 5 minutes.

(c) Indicator solution. Approximately 100 mg. of setopaline C<sup>3</sup> indicator are dissolved in 100 cc. of distilled water. The solution can be used several months for routine analyses. The end-point is slightly sharper, however, when the solution is not more than 2 days old. Until the analyst is accustomed to the end-point, it is well to use a fresh indicator solution.

(d) Stock 0.1377 N solution of ceric sulfate. Approximately 110 gm. of anhydrous ceric sulfate<sup>4</sup> are weighed into an 800 cc. beaker to which 35 cc. of concentrated sulfuric acid and 35 cc. of water are then added. The mixture is stirred and heated, and small amounts of water are added frequently until practically all of the ceric sulfate has dissolved. The solution is filtered, cooled, and the clear solution diluted to 1 liter. Standardization may be carried out by a number of procedures: with ferrous ammonium sulfate (Mohr's salt) (Furman and Wallace, 1930); against sodium oxalate with either the visual or potentiometric end-point (Furman, 1928; Willard and Young, 1928, p. 1322; Treadwell and Hall, 1935); with pure iron (Willard and Young, 1928, p. 1334). We

<sup>3</sup> Setopaline C is manufactured by J. R. Geigy, S. A., Basel, Switzerland, and may be obtained from this company or through Eimer and Amend, New York. The formula of this dye is at present a trade secret. Dr. C. V. Smythe very kindly determined the approximate oxidation-reduction potential of the indicator and obtained a value of +1.1 volts (in 0.1 N HCl).

Alkali-fast green, a dye manufactured by the National Aniline and Chemical Company, Inc., New York, may also be used as the indicator. It is, however, definitely less sensitive than setopaline C.

<sup>4</sup> Anhydrous ceric sulfate is now generally available. The product manufactured by the G. Frederick Smith Company, Columbus, Ohio, is satisfactory.

have found the use of Mohr's salt satisfactory. The method is as follows:

A 0.1 N solution of Mohr's salt,  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ , is prepared by dissolving 39.214 gm. of the salt in 200 to 300 cc. of water, adding 25 cc. of the 18 N sulfuric acid with constant stirring, and finally diluting the clear solution to 1000 cc. *The Mohr's salt must be the highly purified product designed for use as an analytical standard.* In this country satisfactory samples have been obtained from Eimer and Amend, New York, and Mallinckrodt Chemical Works, St. Louis. Kolthoff and Furman (1929) state that the Kahlbaum Mohr's salt, "free from manganese; precipitated by alcohol" is reliable.

15 cc. portions of the ceric sulfate solution are pipetted into 150 cc. beakers, diluted with 50 cc. of water and 3 cc. of 18 N sulfuric acid, and titrated with the standard Mohr's solution until the color of the ceric sulfate is almost bleached out. 8 drops of the setopaline C indicator solution are then added and the titration is continued to a sharp change from golden brown to a light yellow color. No indicator correction is needed for these strong solutions.

The normality of the ceric sulfate solution is calculated as (volume of Mohr's solution/volume of ceric sulfate)  $\times$  0.1000. The strength of the ceric sulfate solution should lie above 0.15 N. From it an exactly 0.1377 N ceric sulfate solution for the permanent stock solution is prepared by dilution with distilled water. *E.g.*, if the normality found as described above is 0.1560, then  $(0.1377/0.1560) \times 1000 = 883$  cc. are diluted to 1 liter to make a 0.1377 N solution. This stock solution is stable for at least 40 weeks when stored in a dark bottle, and 1 liter suffices for about 50,000 sugar determinations.

(e) Dilute ceric sulfate solution for blood sugar titrations. This solution, if kept at room temperature, is prepared the day it is used. If kept at 0°, it can be used for a week. A solution equal to 0.002754 N has been chosen, because each 0.01 cc. is equal to 1 mg. per cent of blood glucose when a 0.1 cc. sample of blood is taken for analysis. To prepare the dilute standard 2 cc. of the 0.1377 N stock solution are delivered from an accurate Ostwald pipette into a 100 cc. volumetric flask, followed by about 5 cc. of the 18 N sulfuric acid. The solution is diluted to the mark with

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distilled water. The added acid makes the  $\text{H}_2\text{SO}_4$  of approximately normal concentration.

The dilute ceric sulfate does not possess the stability of the stronger stock solution. At room temperature no change is measurable in  $1\frac{1}{2}$  hours; a decrease in titer of about 0.5 per cent occurs in 6 hours. However, when kept in the refrigerator, the solution shows only 0.6 per cent decrease in 1 week. Good quality distilled water should be used for the dilutions, because the gradual decreases in titer are probably due partly to reducing impurities in the water. The figures given above were obtained after dilution with the distilled water used routinely in our laboratory.

(f) Acid cadmium sulfate solution. 13.0 gm. of reagent grade cadmium sulfate ( $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ ) and 63.5 cc. of exactly 1 N sulfuric acid are made to 1 liter with distilled water. The sulfuric acid used must be tested for reducing substances as described above under (b).

(g) 1.1 N sodium hydroxide solution. Reagent grade sodium hydroxide "from sodium" must be used to make this solution and the 0.275 N alkali described below. This solution is required only when the macroprecipitation is used.

(h) 0.275 N sodium hydroxide solution. Of the 1.1 N solution 250 cc. are diluted to 1 liter.

(i) 0.275 N sulfuric acid solution, free from reducing impurities.

(j) Barium carbonate powder. This should be of the highest purity obtainable, and free from oxidizing or reducing contaminants.

### *Procedure*

*Precipitation of Blood Proteins*—The microprecipitation is used when only blood sugar is to be determined in the filtrate. If urea also is to be determined, it is convenient to use the macroprecipitation.

*Microprecipitation*—4 cc. of the acid cadmium solution are placed in a  $15 \times 150$  mm. test-tube. Into this solution 0.1 cc. of blood is discharged from a pipette calibrated "to contain."<sup>5</sup> The pipette is rinsed a few times by drawing the cadmium solu-

<sup>5</sup> Folin ((1928) p. 426) has described a suitable technique and pipette for drawing 0.1 cc. blood samples.

tion up into it. When the blood has laked to form a brown solution, one adds 2 cc. of the 0.275 N sodium hydroxide solution and agitates the tube until the contents are uniformly mixed. The tube is then heated in a boiling water bath for 3 minutes, and cooled in running tap water for 2 minutes. Measurement of the cadmium and NaOH solutions with an accuracy of 0.05 cc. is sufficient. It is convenient to heat a number of tubes at the same time, and for this purpose one may use a cylindrical copper rack holding a dozen tubes.<sup>6</sup>

Approximately 0.3 gm. of the barium carbonate powder is now added to each tube;<sup>7</sup> the amount may be measured with sufficient accuracy from a small spoon. The tube is stoppered with the thumb and shaken for 10 to 12 seconds. The mixture is then filtered through a small filter plug of washed cotton<sup>8</sup> into a 50 cc. receiving test-tube<sup>9</sup> and washed with three portions of distilled water, each of 4 cc. The first portion of wash water is shaken a moment with the protein remaining in the precipitation test-tube; the other two are simply run down the inner walls of the tube. It is desirable to have a rack with a double row of holes, so that each receiving tube may stand directly before the corresponding precipitation tube. The washings are conveniently delivered from a 25 cc. graduated pipette with the 4 cc. intervals marked off by a

<sup>6</sup> These copper racks may be obtained from Eimer and Amend, catalogue No. 32002.

<sup>7</sup> If the addition of the barium carbonate is inadvertently omitted, it is still possible to save the analysis by finishing the filtration and washing and adding the alkaline ferrieyanide. This will precipitate the cadmium as a flocculent precipitate of the carbonate which may be filtered off; the few cc. of water required to wash the tube and filter will not materially affect the subsequent reduction or titration.

<sup>8</sup> Good quality absorbent cotton should be washed for 2 days in running tap water and then soaked for 8 hours in several changes of distilled water. The cotton is then dried in a place protected from dust. The amount of cotton used for each filter is about 4 or 5 mg. Filter papers must also be prepared in the same manner, since even the best quality papers may contain large amounts of reducing substances. One must use retentive paper, such as Schleicher and Schüll No. 602 Hard. The funnels used are of 4 cm. diameter; the outflow at the bottom of the cone is contracted to about 3 mm. to hold the cotton plug.

<sup>9</sup> Short, wide test-tubes of 25 or 30 mm. diameter are convenient. We have used 50 cc. Pyrex, round bottom centrifuge tubes of 25 mm. internal diameter.



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colored pencil. Each filter should be allowed to drain almost completely before the next washing is added.

Filtration and washing through cotton require about 20 minutes. Obviously the method is best adapted for a large number of determinations because twenty or thirty filtrations may be carried out simultaneously in this length of time. Where a certain amount of accuracy may be sacrificed for speed, *e.g.* for an emergency sugar analysis, one may employ 7 cm. *washed* filter papers which permit very rapid filtration.

*Macroprecipitation*—This may be used when it is desirable to employ other portions of the filtrate to determine urea. Urea may be determined accurately in the cadmium filtrate by the usual urease or hypobromite methods. Creatinine values on this filtrate are 3 to 7 per cent lower than in corresponding tungstic acid filtrates, non-protein nitrogen about 10 to 20 mg. per cent lower than in tungstate filtrates.

To 1 part of oxalated blood are added 8 parts of the acid cadmium solution, followed by 1 part of 1.1 N sodium hydroxide. About 0.3 gm. of barium carbonate powder is added for each cc. of blood, and the mixture is shaken for a few seconds. It is then centrifuged and filtered through a filter plug of dry washed cotton. A 1 cc. aliquot portion of this filtrate, equivalent to 0.1 cc. of blood, is transferred for analysis to a 50 cc. tube where it is diluted with about 14 cc. of water.

*Reduction of the Ferricyanide*—2 cc. of the alkaline ferricyanide reagent are added from a graduated pipette or burette to the above filtrate, and the tube is gently whirled to mix the solutions. The mixture should be perfectly clear, indicating complete previous removal of cadmium. The tube is heated in a boiling water bath for 15 minutes, then cooled in running water for about 3 minutes. When a number of tubes are heated together, they should be so arranged in a rack or basket that they do not touch each other. A basket with a raised floor is desirable; otherwise, imperfect circulation of the water causes irregular results. It is convenient to heat twelve to eighteen tubes at a time.

*Titration of the Ferrocyanide Produced*—The titration should be performed within 1 hour after the reduction. Just before each solution is titrated, approximately 1 cc. of the 18 N sulfuric acid is added, followed by 7 drops of the indicator. The solution is then

titrated with the 0.002754 N ceric sulfate. The titration must be performed in a white light against a white background. The end-point is a sharp change from golden yellow to golden brown, and can be readily distinguished from a preceding moderate deepening in yellow color. It is best seen when the observer's eye is below the top level of the solution. For ordinary routine analysis one may titrate with a rapid stream of drops until a sharp change occurs. For the titration a burette is used which permits measuring the delivered solution to within 0.01 cc. We employ the Bang microburette (see Peters and Van Slyke (1932), Fig. 1, p. 13).

*Calculation*—The burette reading, in cc. of solution delivered, minus the blank, is multiplied by 100 to give the result directly in mg. of sugar per 100 cc. of blood.

The correction for the blank can be made automatic by using a burette calibrated for 0.4 cc. above the 0 mark. With this type of burette the blank is first determined, and its amount indicated above the 0 point by a fine pencil mark. In subsequent titrations the ceric sulfate solution is started from this mark and the titration carried out as usual. The observed burette reading then indicates the sugar value directly in mg. per cent.

*Blank Determination*—When cotton filters are used in the micro-precipitation, the blank is performed as follows: 2 cc. of 0.275 N sodium hydroxide, 2 cc. of the 0.275 N  $\text{H}_2\text{SO}_4$ , 14 cc. of  $\text{H}_2\text{O}$ , and 0.3 gm. of  $\text{BaCO}_3$  are mixed and shaken for 12 seconds. The suspension is centrifuged, and approximately 15 cc. of the supernatant fluid poured through the usual cotton filter into the 50 cc. receiving tube. 2 cc. of the alkaline ferricyanide are added and the analysis finished as described above. This blank includes all the reagents except the cadmium sulfate. This is omitted because filtration of cadmium hydroxide through cotton in the absence of precipitated blood is very slow, and the cadmium is without influence on the value of the blank.

When washed filter papers are employed, the blank must be carried out with the reagents (including the cadmium sulfate) and the same number of washings as in the determination of blood. Papers from the same batch should be used as in the blood analysis to which the blank is applied.

Our blanks with cotton filters average 0.12 cc. and remain

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perfectly constant for several weeks. Blanks with paper are higher and more variable.

The blank for the macroprecipitation is performed with all the reagents on an amount of water equivalent to the blood sample.

*Stability of Blood Glucose during Preparation of Filtrates*—The blood laked in the acid cadmium solution is stable for 3 hours at 24°. After this time there is a gradual rise in the reducing value because of hydrolysis of protein.

After addition of the sodium hydroxide solution to the acid cadmium-blood mixture, and before the heat coagulation, the samples are stable for at least 4 hours at room temperature, and for 24 hours at 0°.

After the heat coagulation filtration should be begun at once, and the filtrates should be analyzed within an hour after the washing has been completed.

*Permissible Preservatives*—Whole blood preserved by the addition of 10 mg. of sodium fluoride per cc. may be analyzed by this method. We have not tested other preservatives.

### EXPERIMENTAL

*Nature of the Reducing Substance*—It has been shown by Fujita and Iwatake (1931) that the cadmium hydroxide precipitation removes essentially all the non-fermentable reducing substances, in particular glutathione, and that in the filtrate practically the same sugar values are found by ferricyanide reduction as by fermentation. We have been able to confirm these authors and have not found more than 1.5 mg. per cent of residual reducing substance after fermentation of fresh normal human blood with yeast, by the technique of Hiller, Linder, and Van Slyke (1925) and subsequent precipitation of the proteins by the cadmium hydroxide method. Uremic bloods, however, give much higher residual reduction, as shown in Table I.

*Analysis of Blood Containing Added Glucose*—Several samples of human blood containing added pure glucose were analyzed by the microprecipitation method. The results so obtained are given in Table II.

The macroprecipitation gives the same order of precision.

*Comparison with Hagedorn-Jensen Method*—Our method when compared with Hagedorn and Jensen's on ten samples of human

blood with approximately normal sugar contents gave lower values on nine. The average difference was 7 mg. per cent. With blood containing over 150 mg. per cent of sugar, on the other hand, the

TABLE I  
*Non-Fermentable Reducing Substance in Human Blood of Normal and Elevated Urea Content*

Sample No.	Urea N	Non-fermentable reducing substance (as glucose equivalent)
	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
1	12.7	1.5
2	11.7	1.0
3	11.0	1.0
4	10.2	0
5	15.0	1.5
6	37.4	0
7	81.0	12.0
8	100.0	33.0
9	124.0	48.0

TABLE II  
*Determination of Glucose in Human Blood Containing Added Glucose*  
The filtrates were prepared by the microprecipitation method.

Glucose originally present in blood	Glucose added	Glucose found (a)	Glucose calculated (b)	Error $\frac{100(a-b)}{b}$
<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>per cent</i>
79	61	142	140	+1.4
79	120	197	199	-1.0
80	196	277	276	+0.4
70	255	331	325	+1.8
70	317	389	387	+0.5
73	387	448	460	-2.6
73	482	571	555	+2.9
73	642	728	715	+1.8
31*	195	225	226	-0.4

\* Rabbit blood after insulin injection.

Hagedorn-Jensen method gave values slightly lower than our method. We are inclined to believe that the differences here are due to slight errors in the Hagedorn-Jensen method, due to the

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smallness of the excess of ferricyanide present for the higher sugar concentrations.

*Normal Blood Sugar*—Blood samples were taken before breakfast from normal adults, except in a few cases in which they were obtained 4 hours after a low carbohydrate breakfast.

Twenty-three determinations on venous blood gave a mean value of 83 mg. per 100 cc., with a standard deviation of 4 mg. Eighteen analyses of capillary blood averaged 92 mg. per 100 cc., with a standard deviation of 3 mg.

### SUMMARY

A direct titration method for the sugar in 0.1 cc. of blood is described. The blood is deproteinized with cadmium hydroxide, the excess cadmium removed with barium carbonate, and the filtrate is heated with a large excess of ferricyanide. The ferrocyanide produced is titrated by ceric sulfate solution, with setopaline C as indicator.

Only two precise measurements are required, that of the blood sample and that of the final titration. Each 0.01 cc. of 0.002754 N ceric sulfate solution used in the titration indicates 1 mg. per cent of blood sugar.

Except in blood with marked nitrogen retention, the sugar found has agreed closely with the fermentable sugar. Bloods with less than 40 mg. per cent of urea nitrogen have shown only 0 to 1.5 per cent of non-fermentable reducing material.

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## THE LACTONE GROUP OF THE CARDIAC AGLYCONES AND GRIGNARD REAGENT

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The structures of the cardiac aglycones of the digitoxigenin-strophanthidin type, as now well established, are characterized by the possession of a  $\Delta^{\beta,\gamma}$ -lactone side chain on carbon atom (17) of the sterol skeleton. Characteristic of this group is the positive nitroprusside test exhibited by these aglycones.<sup>1</sup> We have shown that this test is unquestionably due to the presence of at least 1 H atom on the  $\alpha$ -carbon atom of the lactone group. In early work from this laboratory, it was also concluded that the presence of such an active hydrogen atom was responsible for the production of methane in the Zerewitinoff-Tschugaeff determination.<sup>2</sup> Such an observation had been made on the dianhydrodilactone,  $C_{23}H_{26}O_4$ , obtained from dianhydrostrophanthidin. This substance, in addition to giving the Legal reaction, gave 1 mole of methane with Grignard reagent. Similarly, in the case of the methyl ester of strophanthidinic acid, 4 moles of methane were obtained.<sup>3</sup> This result indicated, and it was assumed, that 3 moles were due to the three hydroxyl groups and 1 was due to the active H atom of the unsaturated lactone group. It has since been assumed, therefore, as a generalization, that the active hydrogen atom of the  $\Delta^{\beta,\gamma}$ -lactone group of the cardiac aglycones gives 1 mole of methane.<sup>4</sup>

Recently, however, the validity of such a generalization has perhaps been brought in question by the observation of Tschesche

<sup>1</sup> Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **67**, 333 (1926). Jacobs, W. A., Hoffmann, A., and Gustus, E. L., *J. Biol. Chem.*, **70**, 1 (1926).

<sup>2</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **65**, 491 (1925).

<sup>3</sup> Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 811 (1927).

<sup>4</sup> Jacobs, W. A., *Physiol. Rev.*, **13**, 237 (1933). Elderfield, R. C., *Chem. Rev.*, **17**, 208 (1935).



and Haupt<sup>5</sup> who noted that the benzoate of monoanhydroconvallatoxinigenin gives only 2 moles of methane instead of the 3 moles required by two free hydroxyl groups and an active H atom of the  $\Delta^{\beta,\gamma}$ -lactone side chain. This observation appeared to be supported by the similar behavior of the benzoate of anhydrouzarigenin. We have accordingly rechecked our earlier determination on the dianhydrodilactone,  $C_{23}H_{26}O_4$ , from strophanthidin and have extended the determinations to digitoxigenin and its derivatives.

Digitoxigenin was found to give 3 moles of methane which is in accord with the presence of two free OH groups and the active H atom of the  $\Delta^{\beta,\gamma}$ -lactone group. This was confirmed by the determination with digitoxigenin acetate which gave 2 moles of methane due to one free OH group and the  $\Delta^{\beta,\gamma}$ -lactone group. The dianhydrodilactone,  $C_{23}H_{26}O_4$ , again gave 1 mole of  $CH_4$  due to the  $\Delta^{\beta,\gamma}$ -lactone group.

On the other hand, somewhat of an abnormality was experienced in the case of both isomeric anhydrodigitoxigenins.<sup>6</sup> Only 1.4 moles of  $CH_4$  were obtained instead of 2 required by the secondary hydroxyl group and the unsaturated lactone group. Similarly, the tetrahydrodianhydrodilactone obtained by hydrogenation of the above dianhydrodilactone,  $C_{23}H_{26}O_4$ , gave 0.57 mole of  $CH_4$  instead of 1 required by the  $\Delta^{\beta,\gamma}$ -lactone group.

It may be concluded that our original assumption is correct; viz., that the  $\Delta^{\beta,\gamma}$ -lactone group of the cardiac aglycones contains an active hydrogen atom which is not only responsible for the Legal reaction but also gives 1 mole of methane with Grignard reagent. The apparent failure of the benzoates of monoanhydroconvallatoxinigenin and anhydrouzarigenin to react in this sense, as well as the incomplete reactions in the cases of the anhydrodigitoxigenins and the tetrahydrodianhydrodilactone, will require explanation.

#### EXPERIMENTAL

The determinations were made according to the method of Roth.<sup>7</sup> The anhydrous substance was dissolved in 2 cc. of pyri-

<sup>5</sup> Tschesche, R., and Haupt, W., *Ber. chem. Ges.*, **69**, 459 (1936).

<sup>6</sup> Jacobs, W. A., and Elderfield, R. C., *J. Biol. Chem.*, **113**, 611 (1936).  
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<sup>7</sup> Pregl, F., *Die quantitative organische Mikroanalyse*, Berlin, 4th edition, 192 (1935).

dine, and 1 cc. of a solution of methylmagnesium iodide in iso-amyl ether was added. The gas volumes reported are corrected to 0° and 760 mm.

13.070 mg. of the dianhydrodilactone,  $C_{22}H_{26}O_4$ , gave 0.874 cc. of  $CH_4$ , or 1.09 moles. Calculated for 1 mole, 0.800 cc.

13.552 mg. of the tetrahydrodilactone,  $C_{22}H_{30}O_4$ , gave 0.468 cc. of  $CH_4$ , or 0.57 mole. Calculated for 1 mole, 0.821 cc.

6.760 mg. of digitoxigenin gave 1.230 cc. of  $CH_4$ , or 3.04 moles. Calculated for 3 moles, 1.215 cc.

12.985 mg. of digitoxigenin acetate gave 1.418 cc. of  $CH_4$ , or 2.02 moles. Calculated for 2 moles, 1.398 cc.

10.928 mg. of digitoxigenin acetate gave 1.400 cc. of  $CH_4$ , or 2.16 moles. Calculated for 2 moles, 1.178 cc.

13.662 mg. of  $\beta$ -anhydrodigitoxigenin gave 1.243 cc. of  $CH_4$ , or 1.45 moles. Calculated for 1 mole, 0.859 cc.; for 2 moles, 1.718 cc.

14.450 mg. of  $\beta$ -anhydrodigitoxigenin gave 1.262 cc. of  $CH_4$ , or 1.39 moles. Calculated for 1 mole, 0.909 cc.; for 2 moles, 1.818 cc.

11.110 mg. of  $\alpha$ -anhydrodigitoxigenin gave 0.989 cc. of  $CH_4$ , or 1.42 moles. Calculated for 1 mole, 0.699 cc.; for 2 moles, 1.398 cc.



# THE REACTION OF IODOACETATE AND OF IODOACETAMIDE WITH VARIOUS SULFHYDRYL GROUPS, WITH UREASE, AND WITH YEAST PREPARATIONS\*

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It is well established that iodoacetic acid reacts rapidly under physiological conditions, *i.e.* approximate neutrality and a temperature not exceeding 37°, with such sulfhydryl groups as those of cysteine and glutathione (1). It is also known that this reagent can react with other groups, *e.g.* amino groups (2), although this reaction proceeds very slowly, if at all, under physiological conditions. However, because of the possibility of the latter reaction, one cannot conclude that reactions inhibited by iodoacetate involve —SH groups. The possibility exists, however, of considering that reactions which are not inhibited by iodoacetate do not involve —SH groups. The only published results, of which the writer is aware, that contradict such a consideration are those of Hellerman, Perkins, and Clark (3) on urease. These authors present a number of experiments which can be interpreted, and perhaps are best interpreted, on the assumption that the activity of urease is dependent on the presence of —SH groups. They found, however, that iodoacetate did not inhibit urease.

In an effort to determine if there are —SH compounds of known structure which either do not react with iodoacetate or at most react very slowly, we have measured the rate of this reaction for some typical compounds of this class. The results for thioglucose, thiosalicylic acid, cysteine, glutathione, and thio-

\* A preliminary account of these experiments was presented at the Thirtieth annual meeting of the American Society of Biological Chemists at Washington, 1936 (*Proc. Am. Soc. Biol. Chem.*, **8**, xcv (1936); *J. Biol. Chem.*, **114** (1936)).

glycol, at pH 6.1, are given in Fig. 1. For the sake of comparison curves for the reaction of these same compounds with iodoacetamide are also given. It can be seen that the rate of the reaction

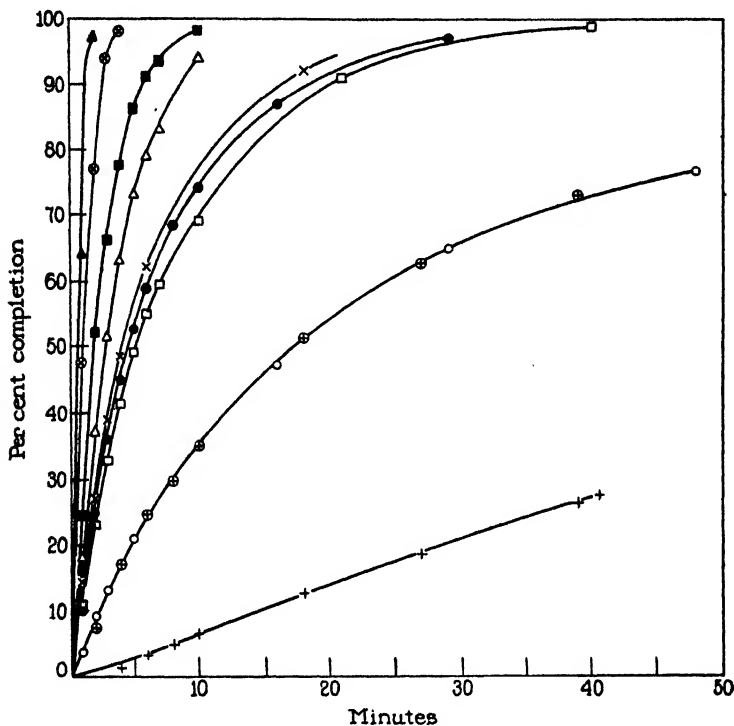


FIG. 1. The rate of reaction of iodoacetate and of iodoacetamide with various sulfhydryl groups at pH 6.1 and 28°. The amount of —SH present was 0.2 cc. of a 0.1 M solution in each case and the amount of iodo compound 0.2 cc. of a 0.5 M solution. The total volume was 1.4 cc. ⊗ represents thiosalicylic acid and iodoacetamide; × thiosalicylic acid and iodoacetate; ▲ thioglucose and iodoacetamide; △ thioglucose and iodoacetate; ■ cysteine and iodoacetamide; □ cysteine and iodoacetate; ● glutathione and iodoacetamide; ○ glutathione and iodoacetate; ⊕ thioglycol and iodoacetamide; + thioglycol and iodoacetate.

for these compounds with either iodo compound is in the order given. It is also evident that in each case the iodoacetamide reacts more rapidly than the iodoacetate. All of these reactions are more rapid at pH 7.1, as can be seen from Table I, which gives

the time required to reach 50 per cent completion. Times of less than 1 minute should be taken to indicate only the order of magnitude of the rate, for the reaction was probably more rapid than the establishment of the gas equilibrium being measured.

Ethyl mercaptan does not react with either of these iodo compounds at a measurable rate, even at pH 8.3 and 37°. This is an example of a simple —SH compound which gives a good nitroprusside reaction and is rapidly oxidized by such oxidizing agents as iodine, but which does not react readily with iodoacetate. Phenyl mercaptan reacts "quite rapidly, but it is difficult to get

TABLE I  
*Reactions of —SH Compounds with Iodo Compounds*

—SH compound	Iodo compound	pH = 7.1		pH = 6.1	
		Time for 50 per cent reaction	Time for iodoacetate + time for iodoacetamide	Time for 50 per cent reaction	Time for iodoacetate + time for iodoacetamide
		min.		min.	
Thioglucose . . . . .	Acetamide	0.57	1.42	0.7	4.15
" . . . . .	Acetate	0.81		2.9	
Thiosalicylic acid . . . . .	Acetamide	0.58	1.45	1.0	4.00
" . . . . .	Acetate	0.84		4.0	
Cysteine . . . . .	Acetamide	0.81	1.38	1.9	2.68
" . . . . .	Acetate	1.12		5.1	
Glutathione . . . . .	Acetamide	1.10	1.82	4.5	3.84
" . . . . .	Acetate	2.0		17.3	
Thioglycol . . . . .	Acetamide	2.0	3.50	17.3	5.40
" . . . . .	Acetate	7.0		93.5	

quantitative results with this compound because of its insolubility. Thiourea reacts slowly even at pH 8.3 and 37°. The reaction of the amino groups of urea, cysteine, glutathione, or guanidine with either of the iodo compounds is too slow under these conditions to be measured in this way.

The effect of these two iodo compounds on the activity of crystalline urease and on commercial Arlco urease is shown in Fig. 2. In agreement with the work cited above (3) these curves indicate that urease is very resistant to the action of iodoacetate, but in addition they indicate that it is quite susceptible to the action of

iodoacetamide. Probably the greatest factor in this rather large difference is the greater reactivity of iodoacetamide as shown in

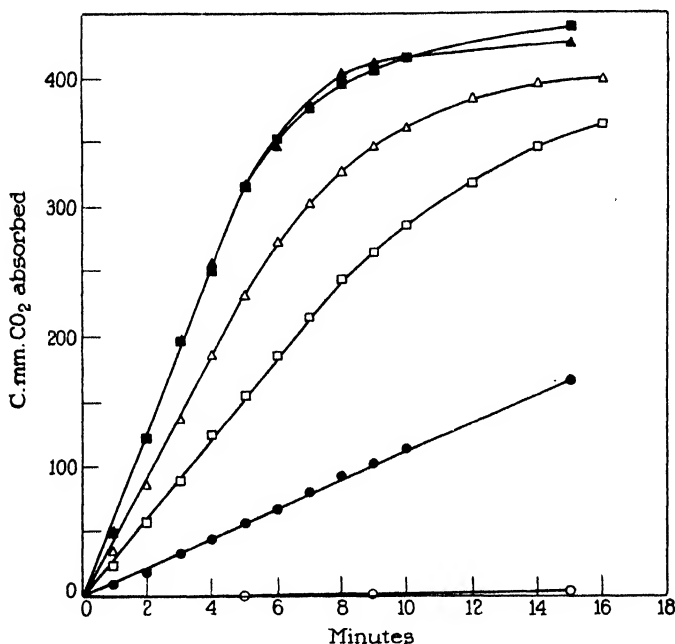


FIG. 2. The effect of iodoacetate and iodoacetamide on urease activity at 28° and pH 7.0. The experiments were carried out with the usual Warburg manometric apparatus. The main room of the vessel contained 1.00 cc. of  $\text{NaHCO}_3$  of such concentration that the final concentration of  $\text{NaHCO}_3$  was 0.314 M, 0.2 cc. of 10 per cent gum arabic, and the amount of enzyme and inhibitor shown. 15 minutes were allowed to elapse from the time these solutions were mixed until the substrate was added from the side arm and the readings started. Unless otherwise stated 0.2 cc. of 0.1 M urea was always added. The gas room contained 100 per cent  $\text{CO}_2$ . ▲ represents  $5 \times 10^{-3}$  mg. of crystalline urease + 0.1 cc. of  $\text{H}_2\text{O}$ ; ■  $5 \times 10^{-3}$  mg. of crystalline urease + 0.1 cc. of 0.1 M  $\text{ICH}_2\text{COONa}$ ; ●  $5 \times 10^{-3}$  mg. of crystalline urease + 0.1 cc. of 0.1 M  $\text{ICH}_2\text{CONH}_2$ ; △ 1.0 mg. of Arleo urease + 0.2 cc. of  $\text{H}_2\text{O}$ ; □ 1.0 mg. of Arleo urease + 0.2 cc. of 0.5 M  $\text{ICH}_2\text{COONa}$ ; ○ 1.0 mg. of Arleo urease + 0.2 cc. of 0.5 M  $\text{ICH}_2\text{CONH}_2$ .

Fig. 1 and Table I, but the similarity of the amide linkage to that of the enzyme substrate, urea, may also play a rôle. That the amide linkage alone is not sufficient to account for the effect of

iodoacetamide is evident from the fact that acetamide, although under our conditions it slightly inhibited urease action, had no comparable effect. Cyanacetamide was also non-effective, as was a mixture of acetamide and iodoacetate.

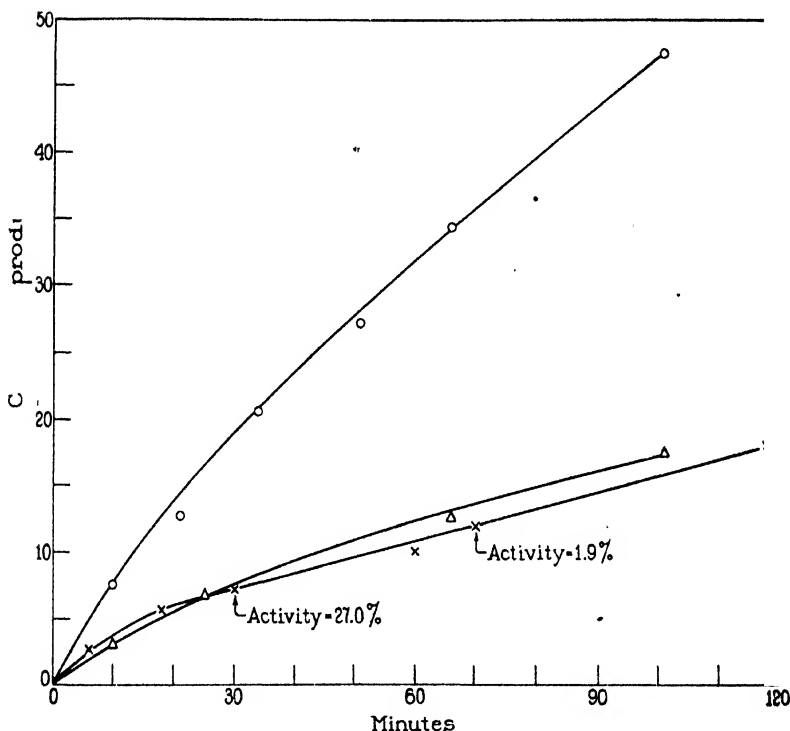


FIG. 3. The reaction of iodoacetate and iodoacetamide with urease at 28°. O represents 100 mg. of Arlco urease + 0.2 cc. of 1.0 M  $\text{ICH}_2\text{CONH}_2$  at pH 7.0, total volume 1.20 cc.,  $\Delta$  100 mg. of Arlco urease + 0.2 cc. of 1.0 M  $\text{ICH}_2\text{COONa}$  at pH 7.0, total volume 1.20 cc.;  $\times$  21.14 mg. of crystalline urease + 0.2 cc. of 0.5 M  $\text{ICH}_2\text{CONH}_2$  at pH 7.0, total volume 2.20 cc. The activity figures given are in per cent of the initial activity.

If either of these iodo compounds reacts with the  $-\text{SH}$  groups of urease, it should be possible to measure this reaction in the same way as with the  $-\text{SH}$  groups just discussed. Fig. 3 shows the results obtained with each compound acting on 100 mg. of Arlco urease. It is evident that the reaction with iodoacetamide



is considerably greater than with iodoacetate. The evidence that the pressure measured here represents a reaction with  $\text{—SH}$  groups is that the amount of pressure corresponds to the decrease of the nitroprusside reaction. As stated above, the inactivation of the enzyme is also considerably greater with the iodoacetamide than with the iodoacetate, so it makes a consistent picture to assume that this inactivation is due to the destruction of these  $\text{—SH}$  groups. The possibility still exists, however, that the inactivation is due to the destruction of some other group and is merely accompanied by a destruction of  $\text{—SH}$  groups, but at the present time we know no such other group.

Fig. 3 also contains a curve for the action of iodoacetamide on crystalline urease (three experiments). It gives a measure of the number of  $\text{—SH}$  groups destroyed at any time. If the total amount of enzyme is known and one can determine the amount of active enzyme at one or more points along this curve, one can then calculate the number of  $\text{—SH}$  groups that have been destroyed per unit weight of enzyme inactivated. Since it would seem logical to assume that if the inactivation is due to the destruction of  $\text{—SH}$  groups one must destroy at least one  $\text{—SH}$  group per molecule, such data should give a minimum value for the molecular weight of the enzyme. According to Sumner and Poland (4), who estimated the amount of  $\text{—SH}$  groups from the strength of the nitroprusside reaction, urease contains one  $\text{—SH}$  group (*i.e.* 32 gm. of sulfur as  $\text{—SH}$ ) per 15,000 gm. This value agrees approximately with the end-value for the iodoacetamide reaction. On this basis one should expect about 30 c.mm. of  $\text{CO}_2$  from the 21.14 mg. of urease used. However, one can see from the curve that the enzyme is 98 per cent destroyed when we have obtained less than half this amount of  $\text{CO}_2$ . Calculated at this point (70 minutes), we have destroyed one  $\text{—SH}$  group per 40,000 gm. of enzyme inactivated. On the assumption that at least one  $\text{—SH}$  group is destroyed per molecule, this gives a minimum value of 40,000 for the molecular weight of the enzyme. This value is probably too low, for, as can be seen, the reaction does not stop at this time, but continues. This continuing reaction undoubtedly overlaps with the enzyme-destroying reaction. This is well illustrated by the result obtained at 30 minutes. At this point we have already inactivated 73 per cent of the enzyme, but have destroyed only one  $\text{—SH}$  group per 49,300 gm. of enzyme

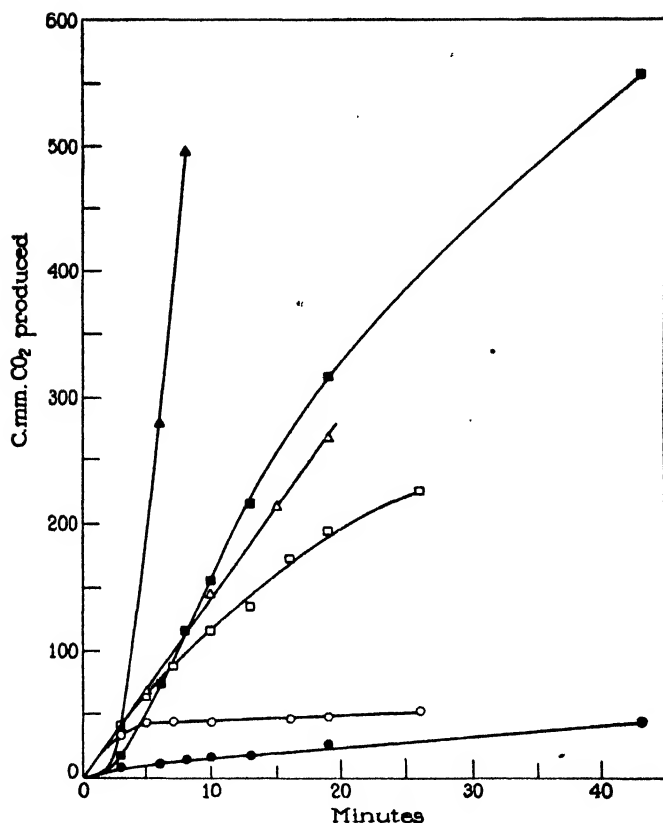


FIG. 4. The effect of iodoacetate and of iodoacetamide on the rate of fermentation by living yeast cells and by cell-free yeast extract at 28°.  $\Delta$  represents the living yeast control. The cells were suspended in  $M/15$   $KH_2PO_4$ . 0.2 cc. of 40 per cent glucose was added from a side arm at time = 0. The total volume was 1.33 cc. The gas room contained 100 per cent  $N_2$ .  $\circ$  same as the control + 0.03 cc. of 0.1  $M$   $ICH_2COONa$ ;  $\square$  same as the control + 0.03 cc. of 0.1  $M$   $ICH_2CONH_2$ .  $\blacktriangle$  yeast extract control. The main room of the vessel contained 0.5 cc. of Lebedev's extract + 0.53 cc. of  $H_2O$ . 0.2 cc. of 40 per cent glucose and 0.02 cc. of 0.15  $M$  potassium hexosediphosphate were added from a side arm at time = 0.  $\bullet$  same as the control + 0.03 cc. of 0.1  $M$   $ICH_2COONa$ ;  $\blacksquare$  same as the control + 0.03 cc. of 0.1  $M$   $ICH_2CONH_2$ . In each case the iodo compound was added 15 minutes before the substrate was added and the readings started.

inactivated. This value is also probably too low for the minimum molecular weight for the same reason as just given. It would

appear then that one can conclude with some assurance that urease can be completely inactivated by iodoacetamide when not more than one-half, and probably not more than one-fourth, of the total  $\text{—SH}$  groups has been destroyed. If we consider that this inactivation is due to the destruction of the  $\text{—SH}$  groups and that the destruction of these groups by any other means would also inactivate the enzyme, the above results lead to the following conclusion. If the enzyme is inactivated by oxidizing the  $\text{—SH}$  groups to  $\text{—S—S—}$  groups, it cannot be reactivated by  $\text{HCN}$ , for this reagent yields one  $\text{—SH}$  group and one  $\text{—SCN}$  group from each  $\text{—S—S—}$  group (5). This is a destruction of one-half the  $\text{—SH}$  groups and this compound will be inactive. The oxidized enzyme can, however, be reactivated by any agent that reduces each  $\text{—S—S—}$  to two  $\text{—SH}$  groups. Thus there is no reason to expect that  $\text{HCN}$  will give the same result as sulfhydryl compounds such as  $\text{H}_2\text{S}$  in reactivating this oxidized enzyme. The same may also be true for some other compounds (6).

If the two iodo compounds are compared as inhibitors of fermentation by living yeast cells, it is found that the iodoacetate is considerably the better, or more correctly the more rapid, of the two (Fig. 4). The same difference holds for the inhibition of oxygen consumption, with either glucose or ethyl alcohol as substrate. This difference is rather surprising in view of the fact that in the  $\text{—SH}$  reactions tested the difference was in the reverse order, and, since one might expect that if penetration of the cell is to limit the action of either, that this would affect the ionized iodoacetate more than the un-ionized iodoacetamide (7, 8). This permeability effect can be avoided by using cell-free extracts and here also the iodoacetate is the more rapid inhibitor of the two (Fig. 4). It is evident, therefore, that the reaction which causes this inhibition is different from the reaction with any of the  $\text{—SH}$  groups tested. We know, at present, no grouping that would react rapidly enough under these conditions to account for the iodoacetate effect and yet react more slowly with iodoacetamide.

#### EXPERIMENTAL

The rate measurements with the iodo compounds and the various  $\text{—SH}$  groups were carried out with the usual Warburg manometric technique. A sodium bicarbonate- $\text{CO}_2$  buffer was used

and the reaction followed by measuring the increase in gas pressure due to the  $\text{CO}_2$  driven out of the liquid by the HI formed from the reaction. 1 molecule of  $\text{CO}_2$  is produced for each molecule of  $-\text{SH}$  that reacts. 100 per cent  $\text{CO}_2$  was used in the gas room and the bicarbonate concentration varied to give the pH desired.

The  $-\text{SH}$  compounds were placed in the main vessel with the

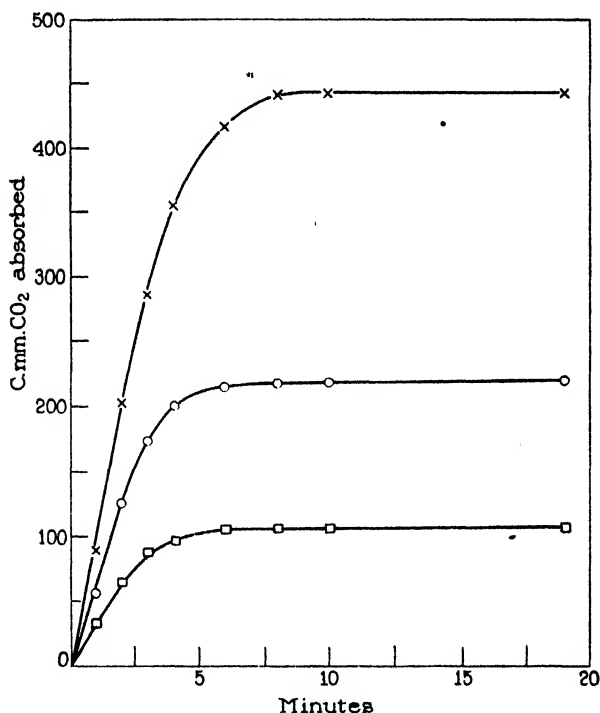


FIG. 5. The pressure obtained with different amounts of urea.  $\times$  represents 0.2 cc. of 0.1 M urea;  $\circ$  0.1 cc. of 0.1 M urea;  $\square$  0.05 cc. of 0.1 M urea. The amount of enzyme was the same in each case.

bicarbonate and the iodo compounds added from a side arm. Controls were run in the same way, without  $-\text{SH}$  compounds to check any pressure change on mixing and any hydrolysis during the experiment. It was found that the amount of hydrolysis under these conditions was negligible. The same amount of  $-\text{SH}$  compound (0.2 cc. of 0.1 M solution) was used in every case, so

the total pressure to be expected was always the same (448 c.mm.). In general the pressure obtained was slightly less than the theoretical. This is due to the autoxidation of the —SH groups during the preparation of the experiment. This difference, if one works rapidly, is never large, but it is different for different compounds, so the results are expressed in per cent of the total pressure obtained in each case.

The crystalline urease was prepared from jack bean meal according to Sumner (9). The crystals were collected by centrifuging, washed, and taken up in distilled H<sub>2</sub>O. The amount of material present was determined by evaporating a portion of the solution to dryness and weighing. The activity of the enzyme was measured manometrically in a bicarbonate-CO<sub>2</sub> buffer by measuring the change in the pressure exerted by the gas confined in the vessel. This pressure diminishes during the reaction due to the absorption of CO<sub>2</sub> into the liquid to neutralize the ammonia set free by the reaction. 1 molecule of CO<sub>2</sub> is absorbed for each molecule of urea split (Fig. 5). The results are expressed in c.mm. of CO<sub>2</sub> (1 gm. molecule = 22,400 cc.). 100 per cent CO<sub>2</sub> was used in the gas room and the bicarbonate concentration was varied to give the pH desired. Readings were made at 1 minute intervals without interrupting the shaking. The urea was always added from a side arm at time = 0.

The error involved in the determination of 1.0 mg. of urea by this method is of the order of 2.0 per cent. For a discussion of the error in the pressure reading see Warburg (10). Furthermore, since ammonia is produced during the reaction and the pH is maintained by the sodium bicarbonate-CO<sub>2</sub> buffer, a part of the ammonia formed will remain as free ammonia. We can calculate the approximate concentration of free ammonia from the equation

$$\frac{(\text{NH}_4^+)(\text{OH}^-)}{(\text{Am})} = K_b$$

The (Am) represents the ammonia present as NH<sub>3</sub> and as NH<sub>4</sub>OH.  $K_b$  is  $1.8 \times 10^{-5}$ . The (OH<sup>-</sup>) is maintained approximately at  $10^{-7}$  M. If we use 1.0 mg. of urea in a volume of 1.5 cc. and consider (NH<sub>4</sub><sup>+</sup>) to be approximately equal to the total ammonia formed, we can calculate (Am) to be equal to

$$\frac{(2.2 \times 10^{-4})(10^{-7})}{1.8 \times 10^{-4}} \quad 1.2 \times 10^{-4} \text{ M}$$

If we express this as c.mm., in keeping with the above, we have 4.0 c.mm. of free ammonia present. This means, of course, that this amount of ammonia has not taken up its equivalent of  $\text{CO}_2$ . The theoretical pressure change to be expected is 374 c.mm., so the error is about 1.1 per cent. The amount of ammonia that will volatilize into the gas room is negligible. From the data given by Bjerrum (11) and that found in Lange (12) one can calculate that under our conditions it will be about 1.0 per cent of the free ammonia present in the solution.

In order that the velocity of the reaction be proportional to the amount of enzyme present it is necessary, since the amount of urea that can be added is limited by the amount of pressure that can be read on the manometer, that one use not more than 0.01 mg. of urease per vessel (total volume of solution about 1.5 cc.). In this dilution in order to get activities as great as those reported by Sumner (9) it is necessary to protect the enzyme from the traces of metal that it is difficult to avoid. The gum arabic recommended by Sumner (13) serves this purpose as does also dinitrosoresorcinol. In order to obtain the readings used in the crystalline urease curve in Fig. 3 it was necessary to use concentrated enzyme solutions (21.14 mg. in 2.0 cc.). The enzyme activity at different times was obtained by removing a sample from this concentrated solution and diluting to the range where the rate of the reaction is proportional to the amount of enzyme present. The inhibiting effect of the iodo compounds was tested both in the presence of and in the absence of the gum arabic. It has no effect on this reaction. The curves shown in Fig. 3 are corrected for retention of  $\text{CO}_2$  (14).

#### SUMMARY

The rate at which the following —SH compounds, thioglucose, thiosalicylic acid, cysteine, glutathione, and thioglycol, react with both iodoacetate and iodoacetamide, has been measured. The rates for the various —SH compounds are in the order given, the thioglucose reacting about 30 times as rapidly as the thioglycol (at pH 6.1). In each case the reaction was more rapid with iodoacetamide than with iodoacetate.

Ethyl mercaptan does not react at a measurable rate with either iodoacetate or iodoacetamide at pH 8.3 and a temperature of 37°.

The fact that crystalline urease is not readily inhibited by iodoacetate is confirmed. Iodoacetamide is found to be a considerably better inhibitor of urease than iodoacetate. The direct reaction of iodoacetamide with urease has been measured. It is concluded that in order completely to inactivate urease by iodoacetamide it is not necessary to destroy more than one-half and probably not more than one-fourth of the —SH groups present.

In contrast to the relative effect of the two iodo compounds on the other reactions studied, iodoacetate inhibits fermentation by living yeast cells and by cell-free yeast extracts more effectively, or more correctly more rapidly, than does iodoacetamide.

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## THE CHEMISTRY OF CRYSTALLINE SUBSTANCES ISOLATED FROM THE SUPRARENAL GLAND

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An investigation of the chemistry of the suprarenal cortex has been under way in this laboratory for the past few years. In December, 1933, a crystalline organic substance was first obtained from an extract of the gland. This substance was used in place of the whole extract of the suprarenal cortex without any other change in experimental conditions (3). The results indicated that the crystalline material possessed physiologic activity similar to that of the extract from which it had been separated. Subsequent investigation has shown that the problem is much more complex than at first realized. We have found several substances which can be separated in crystalline form, all of which appeared to be closely related. It has also been shown that suprarenalectomized dogs and patients with Addison's disease can be maintained in a normal condition without any extract of the suprarenal gland, provided the diet contains adequate amounts of sodium chloride and sodium bicarbonate or citrate (1, 2). A discussion of the physiologic activity of the many crystalline fractions will be deferred until a later publication, but it is now apparent that the crystals first separated do not influence the level of urea in the blood.

Recently Wintersteiner and Pffner (7) and Reichstein (6) have also described the separation and properties of several crystalline fractions from the suprarenal gland. Previous reports on our work have been given at various meetings. We are now presenting in detail the results of our chemical studies which have been carried further than those of Wintersteiner and Pffner and of Reichstein. A comparison of their results with ours is given in the discussion.



Although the details of the method for the preparation of the extract have been changed from time to time, the essential points in the procedure of isolation are briefly as follows: (1) extraction of the minced glands with acetone in the cold, (2) concentration of the extract at low temperature and pressure and extraction of the aqueous concentrate with an organic solvent, (3) fractionation of the material contained in the organic solvent.

It has been recognized for some time that this original crude crystalline material was a mixture of several chemical individuals, so closely related in many of their chemical and physical properties that their complete separation has become a major problem in the investigation. Some success in the isolation of individual compounds has been achieved and degradation products have furnished information as to the components of some mixtures which could not be resolved.

A general description of the fractionation procedures is briefly as follows: The original aqueous concentrate was thoroughly extracted with benzene which was found to be the most suitable organic solvent for this step. The benzene solution was reduced to a small volume and extracted four times with twice its volume of water. The combined aqueous extracts were then reduced to a convenient volume and extracted four times with twice the volume of benzene. This water-benzene, benzene-water distribution was repeated from fifteen to twenty times. This is similar to the procedure of Pfiffner, Wintersteiner, and Vars (5), except that distilled water was used instead of solutions of sodium bicarbonate and hydrochloric acid; also, the process was carried much further.

By this means the crude original extract was separated into three main fractions. Fraction I is much more soluble in benzene than in water and therefore accumulates almost entirely in the first three benzene residues. Fraction II, being somewhat more soluble in water than in benzene, is found in the first four or five aqueous residues; whereas Fraction III possesses about the same solubility in benzene as in water and can be transferred almost quantitatively from one solvent to the other, while Fractions I and II are left behind. Consequently, Fraction III is found in the last aqueous extract of the series of extractions.

Before proceeding to a description of the further treatment of these fractions, which are still mixtures at this stage, consideration

of some of the methods used is in order. Molecular weights were determined chiefly by Rast's camphor method. In order to check the validity of this method for the compounds under consideration, the molecular weight of crystals obtained from Fraction II was determined by the method of Menzies, alcohol being used as the solvent. The results obtained by the two methods were in good agreement. Wintersteiner and Pffner stated that some difficulty was encountered in the use of the camphor method due to decomposition. We have not had this trouble with most of our compounds and Reichstein did not mention any such difficulty. In the case of Compounds A and E, decomposition was noted if the determination was prolonged. However, at least three readings of the melting point could be taken before there was any evidence of decomposition. Since these readings agreed well, the results are taken with reasonable confidence. The formula of Compound E has been established beyond doubt through the dinitrophenylhydrazones and our confidence in the camphor method is well justified.

Since these compounds contain only carbon, hydrogen, and oxygen, the functions of the oxygen atoms can be determined with the aid of the Grignard reagent. This was done in a "Grignard machine" adapted to quantities of 5 to 15 mg. of the substance to be tested. The apparatus is essentially a small scale reproduction of that described by Kohler and Richtmeyer (4), which permits the simultaneous estimation of the number of carbonyl groups and active hydrogen atoms. The determination of active hydrogen atoms is the same as in the usual Zerewitinoff method. The carbonyl groups are estimated by comparison of the total amount of methane evolved on addition of water after the Grignard reagent has acted on the substance with the amount of methane that would be evolved by addition of water to the same amount of pure Grignard reagent. The reagent which has combined with carbonyl groups will not yield methane. The data are expressed as per cent of hydroxyl or of carbonyl. The maximal absolute error in the determination of active hydrogen atoms is estimated as 0.1 cc., and in the determination of carbonyl groups as 0.2 cc. Sufficient material (12 to 15 mg.) was always used so that at least 1 cc. of gas was measured for each active group. When more than one group was present, the accuracy was entirely satisfactory. Even

though a maximal error of 20 per cent was involved, it was easily possible to distinguish between one and two carbonyl groups. The consistency of our results indicates the suitability of this method for the compounds under discussion. Since it is essential that the substance under examination be completely in solution before addition of the Grignard reagent, and since our compounds are very slightly soluble in isoamyl ether, anisole, and various hydrocarbons usually used as solvents in this procedure, pyridine was employed. However, this latter solvent is somewhat objectionable, since it produces a rather large blank in spite of rigid purification. Finally, a mixture of 0.5 cc. of pyridine and 1.0 cc. of isoamyl ether was found to be satisfactory in regard to blank and complete solution of the substances under discussion, and was used for samples up to 15 mg.

The micromethods of Pregl were used for the tests for methoxyl and ethoxyl groups and for ultimate analysis.

### *Fraction I*

This fraction contains two substances, probably more. One of these has been isolated in what is believed to be a pure state. This was accomplished by crystallization of the crude fraction from water. The product of the first crystallization (215 mg.) was a mixture of prisms and rosettes of needles. Recrystallization from water, by allowing the acetone to evaporate from a dilute acetone solution, again yielded a mixture of large prisms and rosettes. The prisms were sufficiently dense to settle rapidly, while the rosettes tended to float. The two forms were thus separated mechanically.

*Compound A*—The prisms weighed 121 mg., but still contained a small amount of the other crystalline form. The melting point was indefinite and measurement of optical activity showed  $[\alpha]_{5461}^{25} = +342^\circ$ . Two more recrystallizations yielded first 100 mg. and finally 77 mg. of prisms which were entirely free of the rosettes. The melting point was then  $177\text{--}179.5^\circ$  (uncorrected) and  $[\alpha]_{5461}^{25} = +347^\circ$  (0.23 per cent in benzene). There was no color test for a double bond with tetranitromethane and no fluorescence in ultra-violet light.

$\text{C}_{26}\text{H}_{36}\text{O}_6$ .	Calculated.	C 72.89, H 8.47, mol. wt. 428
	Found.	" 73.12, " 8.51, " " 410

Oxidation of these prisms produced an acid which had been encountered several times previously on oxidation of various crude non-crystalline fractions. The material (88 mg. of Compound A) was dissolved in 15 cc. of acetone. To this was added a mixture of 25 cc. of a 2.0 N solution of potassium dichromate, 7 cc. of 5 N sulfuric acid, and 18 cc. of water. The oxidation was allowed to proceed  $1\frac{1}{2}$  hours at room temperature. After removal of the acetone under reduced pressure the reaction mixture was thoroughly extracted with ether. The acid was extracted from the ether with a solution of sodium carbonate. An insoluble, highly crystalline acid (35 mg.) was precipitated from the aqueous solution upon acidification with hydrochloric acid. There remained in the ether 40 mg. of a non-acid having  $[\alpha]_{5461}^{25} = +257^\circ$  (0.06 per cent in benzene). This, on retreatment with chromic acid, yielded a second crop of the acid. The acid was recrystallized from hot water. It does not have a true melting point, but decomposes over a considerable range of temperature ( $245\text{--}260^\circ$ ). When it was tested with tetranitromethane, a strong yellow color was produced;  $[\alpha]_{5461}^{25} = +290^\circ$  (0.1 per cent in 30 per cent alcohol).

$\text{C}_{20}\text{H}_{26}\text{O}_4$ . Calculated, C 72.68, H 7.93; found, C 72.58, H 8.07

Previous samples of an acid which decomposed over the range of  $240\text{--}260^\circ$  and which were obtained by the oxidation of crude material had much the same properties. The analytical values lay between the values calculated for  $\text{C}_{20}\text{H}_{26}\text{O}_4$  and those calculated for  $\text{C}_{20}\text{H}_{28}\text{O}_4$ . The test with tetranitromethane was usually weak. Repeated crystallizations of a large sample of this kind yielded a product, the analysis of which gave values which agreed well with those calculated for  $\text{C}_{20}\text{H}_{28}\text{O}_4$ . It gave no color with tetranitromethane;  $[\alpha]_{5461}^{25} = +267^\circ$  (0.10 per cent in 30 per cent alcohol).

$\text{C}_{20}\text{H}_{28}\text{O}_4$ . Calculated. C 72.24, H 8.49  
Found. " 72.55, 72.32, " 8.38, 8.24

In another case, a sample of this type of acid was obtained by chromic acid oxidation of amorphous material, and decomposed at  $248\text{--}260^\circ$ . After several recrystallizations from dilute alcohol the analysis of the acid agreed with the values calculated for  $\text{C}_{20}\text{H}_{28}\text{O}_4$ . The molecular weight was determined by titration; 27 mg. required 8.2 cc. of 0.01 N sodium hydroxide.

$C_{20}H_{26}O_4$ .	Calculated.	C 72.68,	H 7.93,	mol. wt. 330
	Found.	" 72.38, 72.54,	" 8.07, 7.93,	" " 329

This sample gave a strongly positive test with tetranitromethane. It proved to contain 1 active hydrogen atom and two carbonyl groups.

$C_{20}H_{26}O_4$ . Calculated, 1OH 5.15, 2CO 17.0; found, OH 5.46, CO 16.9

For future reference we shall designate the acid,  $C_{20}H_{26}O_4$ , as Acid 1 and  $C_{20}H_{28}O_4$  as Acid 2.

*Compound B*—In most cases, however, in which the oxidation was carried out on crude amorphous material, the analyses of the products indicated mixtures of  $C_{20}H_{26}O_4$  and  $C_{20}H_{28}O_4$ . Since Compound A is the precursor of the acid  $C_{20}H_{26}O_4$ , it is possible that the substance (Compound B) obtained in this same fraction and which crystallizes in the form of rosettes of needles possessing a melting point of 135–139° (uncorrected) is the precursor of the acid  $C_{20}H_{28}O_4$ . As yet, not enough of this compound has been obtained sufficiently pure for an investigation of this possibility. Analysis of this material showed the following.

$C_{24}H_{34}O_6$ .	Calculated.	C 71.23, H 8.98
$C_{24}H_{34}O_6$ .	"	" 71.59, " 8.48; found, C 71.55, H 8.76

Both of these  $C_{20}$  acids are quite resistant to further oxidation with chromic acid. On the other hand, they are easily attacked by alkaline silver solutions.

### Fraction II

The crude crystals generally contained some amorphous material which was best removed by solution in acetone and precipitation with petroleum ether. The crystals obtained by this procedure were invariably needles, usually grouped in rosettes. The needle form could be changed to octagonal plates by crystallization from dilute alcohol, although sometimes the needle form also separated from this solvent. The crystals thus obtained varied in composition. The carbon content varied from 68.0 to 69.5 and the hydrogen from 8.8 to 9.5 per cent. The material was subjected to

various schemes of fractionation with only partial success; that is, it was not possible to isolate chemical individuals from the mixture. However, after oxidation with alkaline silver it was possible to isolate in pure form an acid having the composition  $C_{21}H_{34}O_6$ , which we shall call Acid 3. The yield of this acid and the optical activity of the crude material were used to follow the fractionation. The most successful method involved solution in acetone, addition of a relatively large volume of water, removal of the acetone *in vacuo*, and finally evaporation of the water *in vacuo*. When the acetone was all removed a portion of the material separated promptly (Subfraction II, a). As the water was evaporated further separation of crystals occurred (Subfraction II, b). The evaporation was interrupted from time to time and the crystals removed. The volume was finally reduced to about 25 cc. and the portion which remained dissolved was designated as Subfraction II, c. This procedure was repeated with the various fractions until their properties ceased to change.

*Subfraction II, a, Compound C*—This fraction gave the largest yield of Acid 3 and possessed the highest specific rotation. It was further fractionated from acetone and petroleum ether (1:1) and, although the less soluble portion yielded a larger proportion of acid, the optical activity was not changed appreciably. The maximal yield of Acid 3 was 45 per cent and could not be increased by further fractionation. The precursor of Acid 3 will be designated as Compound C. Although not actually isolated, some of its properties are well characterized and this designation will facilitate discussion.

The melting point of the best product was 220–227° (uncorrected) with decomposition. The optical activity varied greatly with the solvent. In alcohol  $[\alpha]_{5461}^{25} = +110^\circ$  (0.087 per cent), while in benzene  $[\alpha]_{5461}^{25} = +56^\circ$  (0.06 per cent). The solubility in water was about 0.025 per cent; in benzene, about 0.06 per cent; it was readily soluble in acetone and alcohol.

Other samples of this fraction varied somewhat in properties. Analyses indicated a mixture of  $C_{21}H_{34}O_6$  and one or more other compounds having slightly different carbon and hydrogen contents. The molecular weight was determined by Rast's camphor method and by Menzies' method.

Method	Substance	Camphor	$\Delta t$	Molecular weight	
				Found	Calculated for $C_{15}H_{24}O_5$
Camphor . . . . .	3.7 mg.	39.4 mg.	10.5°	358	366
		Alcohol	$\Delta p$		
Menzies' . . . . .	0.2203 gm.	22.8 cc.	22.0 mm.	370	366

Determinations of active hydrogen atoms and carbonyl groups showed the presence of three hydroxyl groups, one carbonyl group, and 1 inactive oxygen atom. The inactive oxygen atom is presumed to be involved in an ether linkage. The test for a methoxyl or ethoxyl group was negative.

$C_{15}H_{24}O_5$ .	Calculated.	3OH 13.9, 1CO 7.65
Sample 1.	Found.	OH 13.7, CO 6.1
" 2.	"	" 13.4, " 7.1

The oxidation of this fraction with alkaline silver was usually carried out as follows: to 100 mg. dissolved in 5 to 10 cc. of alcohol was added a solution of 625 mg. of silver sulfate in 8 cc. of 2 N ammonium hydroxide and 8 cc. of N sodium hydroxide solutions. After a slight lag the oxidation proceeded rapidly and was complete within 45 minutes. The consumption of silver was 2.3 to 2.9 equivalents for each mole, calculated for a molecular weight of 366. The metallic silver was filtered out and washed well with 50 per cent alcohol. The excess silver ions in the filtrate were precipitated with hydrochloric acid. The filtrate was then made alkaline with sodium carbonate and the alcohol was removed *in vacuo*. The aqueous residue was extracted with ether to remove a small amount of non-acid. After acidification to Congo red and saturation of the solution with sodium chloride the acid was extracted with ether. Assuming a molecular weight of 366 the yield was 90 to 100 per cent. The dry product was leached with small portions of cold acetone. Up to 42 per cent of the material (Acid 3) remained undissolved. Addition of several volumes of petroleum ether to the acetone solution precipitated material which, on further treatment with cold acetone, yielded enough Acid 3 to raise the total yield of this acid to 45 per cent.

*Acid 3*—The acetone-insoluble portion was dissolved in hot ethyl acetate with the aid of a little alcohol. On standing overnight long blades were deposited. For analysis Acid 3 was recrystallized to a constant melting point. It is sparingly soluble in hot acetone, cold dioxane, and petroleum ether; readily soluble in water and alcohol. It decomposes sharply at 240–242° (uncorrected). Under ultra-violet light it fluoresces strongly with an almost white light. It is slowly attacked by alkaline silver and does not give a color with tetranitromethane. The molecular weight was determined by titration. A sample of 45.6 mg. required 11.75 cc. of 0.01 N NaOH.

$C_{21}H_{34}O_6$ .	Calculated.	C 65.92,	H 8.96,	mol. wt. 382
	Found.	" 66.03, 65.86,	" 9.02, 9.09,	" " 388
	Calculated.	3OH 13.3, 4OH 17.8		
	Found.	OH 17.3		

*Oxidation of Acid 3, Ketone 1*—Acid 3 is readily oxidized by chromic acid to a ketone. The acid (50 mg.) was dissolved in water (10 cc.) as the sodium salt. To this was added a 2 N solution of potassium dichromate (2 cc.) and N sulfuric acid (5.5 cc.). A flocculent non-crystalline precipitate appeared but disappeared as the oxidation proceeded. In this instance 4 equivalents of oxidizing agent were used in 1 hour. 12 mg. of acid and 34 mg. of a non-acid were obtained. The non-acid melted at 159–160° (uncorrected) with slight softening at 156° after recrystallization from water.

$C_{20}H_{30}O_3$ .	Calculated.	C 75.41,	H 9.50
	Found.	" 75.36, 75.42,	" 9.52, 9.59

*Subfraction II, b*—In addition to its greater solubility in water, this fraction differs from Subfraction II, a in that, when treated with alkaline silver under the same conditions, it gives not more than a 20 per cent yield of Acid 3.

At first the total acid obtained from the silver oxidation of this fraction was treated with chromic acid under the same condition as was Acid 3. The non-acid recovered from the oxidation crystallized from water as a mixture of two crystalline forms—needles and plates. This mixture melted at 142–145° with decomposition. The analyses of several such preparations were not in very good agreement, indicating a mixture.



Inasmuch as oxidation of Fraction II, b always gave some Acid 3 (10 to 20 per cent), this was removed after oxidation with silver through its insolubility in acetone before proceeding with further study of the acetone-soluble fraction. Apparently this is practically identical with the acid fraction derived from the silver oxidation of Subfraction II, a which is soluble in acetone.

Its properties suggest that it is a mixture. Part of it easily forms an amorphous lactone which is resistant to oxidation with chromic acid under the conditions used for the oxidation of Acid 3. However, the portion which does not close to a lactone can be oxidized to a ketone with chromic acid. Thus far attempts to crystallize this acid or mixture of acids have failed for the most part. In one case, a small amount of crystalline material was obtained from this fraction. It is not certain that this is representative of the portion which does not form a lactone.

$C_{10}H_{12}O_4$ . Calculated, C 65.17, H 8.76; found, C 64.98, H 8.76

*Oxidation of Acid Fraction Soluble in Acetone from Subfraction II, b, Lactone Formation*—Several preparations of the acid fraction soluble in acetone have been oxidized with chromic acid. A typical result is as follows: From 84 mg. of Subfraction II, b ( $[\alpha] = +104^\circ$ ) there were obtained by oxidation with silver under the conditions previously described, 78 mg. of acid. This yielded only 8 mg. of Acid 3. The remainder was easily soluble in less than 1 cc. of cold acetone. To this acid fraction in 5 cc. of methyl alcohol and 1.6 cc. of water, 1 cc. of 5 N sulfuric acid was added. After standing for 5 minutes the alcohol was removed *in vacuo* and the aqueous residue was extracted with ether. The ether extract was washed with sodium carbonate solution and then with water. The ether contained 39 mg. of a non-acid and 30 mg. of acid were recovered from the sodium carbonate solution after acidification and extraction with ether. The treatment with mineral acid, therefore, had converted more than half of the acid originally present into a lactone. Each of these fractions was treated separately with chromic acid under the conditions used for the oxidation of Acid 3. The non-acid fraction yielded 25 mg. of material which was apparently unchanged. On heating with 0.1 N sodium hydroxide for a half hour it required 0.7 cc. of 0.1 N base.

*Ketone 2*—With chromic acid the acid fraction (30 mg.) gave a

nearly quantitative yield of needle-like crystals. This material was non-acid, was not affected with sodium hydroxide, and melted, after two recrystallizations from water, at 159–161° (uncorrected). It forms a crystalline semicarbazone.

$C_{15}H_{25}O_5$ . Calculated, C 74.94, H 9.27; found, C 75.03, H 9.19

Further work is under way to determine the nature of this entire acetone-soluble acid fraction.

*Subfraction II, c, Compound D*—This fraction consists largely of a polyalcohol or possibly a mixture of two such alcohols, which can be separated by heating the mixture with dilute sodium hydroxide. Since the compounds of Subfraction II, b, as well as of Subfraction II, a, are completely converted to acids by this treatment, the polyalcohol, which is stable to sodium hydroxide, can be further separated by extraction of the alkaline solution with ether. It is characterized by its relatively low solubility in cold acetone and high solubility in water. Repeated crystallization from hot acetone gave a product which crystallized in hexagonal plates and melted at 214–216° (uncorrected). This is undoubtedly the same material described by Wintersteiner and Pffner as Compound A and by Reichstein as Substance A. Some of the properties and the analyses show considerable discrepancies, however. Our best preparation gave an analysis which agreed very well with the values calculated for  $C_{20}H_{36}O_6$ . Other preparations, which had melting points of 213–215° after preliminary softening at about 170°, had lower carbon contents. It was thought that this was due to the presence of hydrated crystals and that the partial melting at about 170°, also observed by Wintersteiner and Pffner and by Reichstein, was caused by the presence of this hydrate which lost water at that temperature and consequently showed the same final melting point as the anhydrous material. The experiment to be described later supports this hypothesis, but the decidedly higher carbon content of Reichstein's product and the agreement in composition of his samples dried at 100° and 170° are contrary to this conclusion. Lack of material has prevented us from settling this point at present.

Our best sample (Compound D) was derived from 379 mg. of crude crystalline material. It was decolorized in hot acetone solution with charcoal and recrystallized to a constant melting

point;  $[\alpha]_{461}^{25} = +29^{\circ}$  (0.13 per cent in acetone). It crystallized from hot acetone as large hexagonal prisms. This particular sample did not show a preliminary softening at about  $170^{\circ}$  but melted at  $214\text{--}216^{\circ}$  (uncorrected). It was dried to constant weight at  $80^{\circ}$  and 0.1 mm. over anhydrous magnesium perchlorate and was then very hygroscopic.

$\text{C}_{20}\text{H}_{34}\text{O}_6$ .	Calculated.	C 67.74,	H 9.67, 4OH 19.2
$\text{C}_{20}\text{H}_{38}\text{O}_6$ .	"	" 67.36,	" 10.18
	Found.	" 67.27, 67.33,	" 10.25, 10.00, OH 19.5

4 active hydrogen atoms were found; there was no evidence of a carbonyl group in the quantitative estimation, and the material was recovered unchanged after treatment in aqueous solution with a solution of dinitrophenylhydrazine in 2 N hydrochloric acid. The test with tetranitromethane was negative.

The following experiments create uncertainty as to the number of hydrogen atoms, but support the conclusion that there are 20 carbon atoms.

About 15 mg. of the crystals which were analyzed were dissolved in a few drops of warm alcohol. The solution was diluted to 15 cc. with water and allowed to stand 48 hours. The solution was perfectly clear. Then 1 cc. of concentrated hydrochloric acid was added. In a short time the solution became cloudy and crystals soon appeared. The separation of crystals was rather slow but was complete in 24 hours. These crystals were large blades. After being dried in the air they melted at  $145\text{--}155^{\circ}$  with vigorous evolution of gas. Drying for 3 hours at  $80^{\circ}$  and 0.1 mm. did not change the melting point, but after the substance was crystallized from acetone the melting point was  $165\text{--}170^{\circ}$  with decomposition. This was repeated with 19.0 mg. of material. The solution in 2 cc. of alcohol was diluted to 21 cc. with water and 1 cc. of concentrated hydrochloric acid was added. After standing 4 days, there were recovered 15.0 mg. of crystals dried to constant weight at  $80^{\circ}$  and 0.1 mm. over anhydrous magnesium perchlorate. This material was not hygroscopic. It melted at  $150\text{--}155^{\circ}$  (uncorrected).

$\text{C}_{20}\text{H}_{34}\text{O}_6 \cdot \frac{1}{2}\text{H}_2\text{O}$ .	Calculated.	C 66.07,	H 9.71
	Found.	" 65.99, 66.07,	" 9.82, 9.86

When tested with tetranitromethane, a yellow color was produced. After the material had been dried at  $170^{\circ}$  and 0.1 mm., the test was negative.

In another experiment 50 mg. of the alcohol dissolved in 0.5 N sulfuric acid were heated for 2 hours on the steam bath. The product thus obtained was similar to that just described; m.p.  $172\text{--}178^{\circ}$  (uncorrected).

$\text{C}_{20}\text{H}_{34}\text{O}_5 \cdot \frac{1}{2}\text{H}_2\text{O}$ . Calculated. C 66.07, H 9.71

$\text{C}_{20}\text{H}_{36}\text{O}_5 \cdot \frac{1}{2}\text{H}_2\text{O}$ . " " 65.70, " 10.21; found, C 65.86, H 9.72

In the meantime most of the material had been used for other purposes and a settlement of the uncertainties was not possible. For the present we conclude that the substance described possesses the formula  $\text{C}_{20}\text{H}_{34}\text{O}_5$  or  $\text{C}_{20}\text{H}_{36}\text{O}_5$ , that it forms a very stable hydrate which contains a half molecule of water, and that this can be removed by heating above  $155^{\circ}$  or by repeated crystallization from hot acetone. There are four hydroxyl groups in the molecule and 1 inactive oxygen atom. Further work on this substance is in progress.

Oxidation of this alcohol with chromic acid yielded Ketone 3. The alcohol (140 mg.) was dissolved in 10 cc. of acetone and 5 cc. of water. To this solution were added 6 cc. of 2 N potassium dichromate solution and 2 cc. of 5 N sulfuric acid. This mixture was allowed to stand for  $2\frac{1}{2}$  hours at room temperature. At the end of this time almost 10 equivalents of oxidizing agent had been consumed. A small portion of the product was acidic and was discarded. The crude non-acid portion weighed 100 mg. This was recrystallized from dilute alcohol. The first crop weighed 53 mg. and consisted of blade-like needles which melted at  $160\text{--}161.5^{\circ}$  (uncorrected);  $[\alpha]_{\text{D}}^{25} = +229^{\circ}$  (0.074 per cent in benzene). The second crop of 30 mg. melted at a lower temperature and was obviously impure. The first fraction was dried for 2 hours at  $80^{\circ}$  and 0.1 mm. over anhydrous magnesium perchlorate. Analysis speaks for the composition  $\text{C}_{18}\text{H}_{24}\text{O}_3$  (Ketone 3).

$\text{C}_{18}\text{H}_{24}\text{O}_3$ . Calculated. C 74.94, H 8.40

$\text{C}_{18}\text{H}_{26}\text{O}_3$ . " " 74.48, " 9.03

Found. " 74.92, 75.06, " 8.57, 8.84

The determination of hydroxyl and carbonyl groups showed the presence of one of each. The third oxygen atom was inactive.

$C_{11}H_{14}O_3$ . Calculated, 1OH 5.90, 1CO 9.7; found, OH 6.41, CO 11.3

The ketone did not give an iodoform test. 25 mg. yielded 15 mg. of an oxime which crystallized in rosettes of needles and melted with final decomposition over the range 205–232°. Difficulties encountered in the attempt to purify the oxime prevented a more definite characterization of this derivative.

### Fraction III

*Compound E*—On concentration of the aqueous solution containing Fraction III several crops of crystals were obtained. The least soluble portion of the crude material usually had a specific rotation of about +200° in benzene. The specific rotation was raised as high as +257° by repeated crystallizations from hot benzene but could not be raised further. It was found that the material still contained some of Compound D which had been carried through in the partition procedure. The specific rotation was raised to a maximum by repeated crystallization from water. The material was dissolved in acetone and this solution diluted with water. As the acetone evaporated, crystals separated. Three crystallizations were sufficient to yield a single substance. A fourth treatment caused no change in optical activity;  $[\alpha]_{5461}^{25} = +269^\circ$  (0.125 per cent in benzene). The melting point was 201–208° (uncorrected) with decomposition. The analysis and the molecular weight determination were in excellent agreement with the formula  $C_{21}H_{30}O_5$ . This was confirmed by analysis of the dinitrophenylhydrazone. The substance was dried 2 hours at 110° and 0.1 mm. over anhydrous magnesium perchlorate.

$C_{21}H_{30}O_5$ . Calculated.	C 69.57,	H 8.35,	mol. wt. 362
Found.	" 69.82, 69.60,	" 8.37, 8.72,	" " 364

The 2,4-dinitrophenylhydrazone proved to be a well characterized derivative which easily could be obtained analytically pure. It is so easily separated and recognized that it has been used for the determination of Compound E in certain mixtures. For its preparation 25.0 mg. of Compound E were dissolved in 4 cc. of alcohol. To this solution were added 10 cc. of a saturated solution

of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid. Almost immediately a red gelatinous precipitate appeared. After standing several hours the precipitate was centrifuged out and washed once with 25 per cent alcohol. It was dissolved in a hot mixture of 6 cc. of ethyl acetate and 10 cc. of alcohol; 5 cc. of water were added while hot and the solution allowed to stand overnight. The red-orange crystals weighed 29 mg. and after recrystallization from ethyl acetate melted at 255–256° (uncorrected) with decomposition.

$C_{17}H_{14}O_4N_4$ .	Calculated.	C 59.75,	H 6.32,	N 10.33
	Found.	" 59.90, 59.62,	" 6.10, 6.40,	" 10.24

The dinitrophenylhydrazone is sparingly soluble in cold alcohol and ethyl acetate. By crystallization from a large volume of hot ethyl acetate it can be separated readily from impurities.

Compound E shows selective absorption with a maximum at 2370 Å. The molecular extinction coefficient at 2370 Å. was 16,150.<sup>1</sup> Absorption in this region by their active hormone fraction has been noted by Pfiffner, Wintersteiner, and Vars. Reichstein observed that his Substance C showed a band with maximal absorption at about 2350 Å. As has been pointed out by these workers, such an absorption band is indicative of an  $\alpha,\beta$ -unsaturated ketone grouping. The nature of this compound is under investigation.

#### DISCUSSION

The properties of the compounds are summarized in Table I. The similarities of Compounds C, D, and E are obvious. Compound C, although not isolated, deserves some further comment, since the information gained from its oxidation permits a few conclusions as to its structure. The oxidation to an acid with alkaline silver speaks for the presence of an aldehyde group. We are not unaware of the possibility of a rearrangement in the alkaline medium but this seems unlikely. When the crystals of Subfractions II,a and II,b are heated with 0.05 N sodium hydroxide,

<sup>1</sup> We are indebted to Professors Koch and Hogness of the University of Chicago for the determination of the absorption spectrum and to the Rockefeller Foundation which is supporting spectroscopic biological investigation at the University of Chicago.

TABLE I  
Composition and Properties of the Compounds and Their Oxidation Products

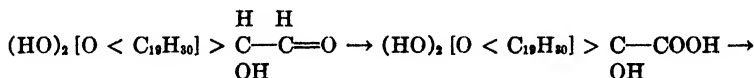
	Compound			Acid			Ketone		
	Formula	M.p (uncorrected) °C.	$[\alpha]_{5461}^{25}$ degrees	No.	Formula	M.p. (uncorrected) °C.	$[\alpha]_{5461}^{25}$ degrees	No.	Formula
A	$C_{20}H_{30}O_5$	177-179.5	347	1	$C_{20}H_{30}O_4$	*	290		
B	$C_{20}H_{30}O_5$	135-139		2†	$C_{20}H_{30}O_4$	*	267		
C	$C_{21}H_{34}O_5$	Separate as mixed crystals		3	$C_{21}O_{14}O_6$	240-242		1	$C_{20}H_{30}O_3$
				4†	$C_{20}H_{32}O_6$			2†	$C_{18}H_{28}O_3$
D	$C_{20}H_{34}O_5$ or $C_{20}H_{36}O_5$	214-216	29					3	$C_{18}H_{24}O_3$
E	$C_{21}H_{36}O_5$	201-208	269						

\* Neither of these two acids has a definite melting point or decomposition point. They begin to shrivel and turn brown at 245° and finally evolve gas at 260° when the temperature is raised at the rate of 5° a minute.

† The relation of Acid 2 to Compound B is a matter of speculation at present. They are grouped together for convenience.

‡ It is not known whether Ketone 2 is derived from Acid 4. They are placed on the same line since both are derived from the substance or substances which accompany Compound C.

they are quantitatively converted to a mixture of acids among which there is none of the acid (Acid 3) produced by the oxidation of Compound C with alkaline silver. This conversion to acid with alkali alone does not occur to an appreciable extent in the cold. The degradation of the acid,  $C_{21}H_{34}O_6$ , to the ketone,  $C_{20}H_{30}O_3$ , leads to the conclusion that there is a tertiary alcohol group adjacent to the aldehyde group. Since only 1 carbon atom but 3 oxygen atoms were lost, a molecule of water must have been eliminated with formation of a double bond. This was confirmed by a positive, though not very strong, test with tetranitromethane. Assuming that the inactive oxygen atom is an ether oxygen, we may write the following partial formulas for Compound C and its oxidation products.



This interpretation is somewhat different from the one quoted by Reichstein. Our previous interpretation was based on the preparation of a ketone,  $C_{19}H_{30}O_3$ , and its semicarbazone. The ketone was thought to be derived from the acid  $C_{21}H_{34}O_6$  before the nature of the mixture from which this acid came was fully understood. It is now evident that the side chain which is eliminated in the ketone consists of 1 carbon atom instead of 2.

A comparison of our compounds with those of Wintersteiner and Pfiffner and of Reichstein reveals agreement only in the case of our Compound D. The other substances which we have isolated have no counterpart in those described by the other workers. Several of our preparations have had compositions identical with that of a compound,  $C_{21}H_{34}O_5$ , as given by Reichstein for his Substances C, D, and E, but the oxidation with alkaline silver revealed them as mixtures. His Substance G has an absorption band and a high specific rotation in common with our Compound E, but the melting point and composition are not at all similar. We have also had many crystalline preparations with the properties and composition of the Compound B of Wintersteiner and Pfiffner but they have invariably proved to be mixtures.

These other workers have recognized the difficulties involved in



the separation of individuals from the mixture of compounds present in the extracts of the suprarenal gland and the possibility that some of the "compounds" may in fact be mixtures. Portions of their analytical data suggest that that is the case.

Our experience in heating crystalline preparations with 2,4-dinitrophenylhydrazine in various media was similar to that of Wintersteiner and Piffner; no definite products could be isolated. However, treatment in the cold has yielded crystalline dinitrophenylhydrazones from which several individuals have been isolated. This derivative of Compound E has been described.

The test for a double bond with tetranitromethane has been used with some success, but there was one case of a false positive test which should be pointed out. When Compound D was crystallized from approximately N hydrochloric acid, it gave a strongly positive test which at first was interpreted to mean that a double bond had been produced through loss of a molecule of water. The analysis, however, was not compatible with this interpretation. When the material was dried at 170° and 0.1 mm., the residue did not give a test with tetranitromethane. Evidently the water of hydration caused the development of the yellow color. We have not seen any previous mention of such a false indication of the presence of a double bond, although negative tests have been reported when a double bond was undoubtedly present.

#### SUMMARY

The fractionation of an extract of the suprarenal gland has been described briefly. Four compounds have been isolated and a fifth has been identified through its products of oxidation. These compounds, which contain only carbon, hydrogen, and oxygen, are closely related in physical and chemical properties and are accompanied by other closely allied compounds which have not yet been isolated in a pure state. Their general nature is that of polyhydroxycarbonyl compounds. Some of the details of their structures have been determined. A possible error in the application of the tetranitromethane test for double bonds has been discussed.

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## STUDIES IN MUSCULAR DYSTROPHIES

### THE PRESENCE OF SIMPLE GUANIDINE DERIVATIVES IN THE URINE

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In studies on muscular dystrophy in young individuals investigations of the urine were made along three lines, as follows: (a) the estimation of creatinine and creatine, according to Folin (1) and Benedict (2) respectively; (b) the study as to whether the creatine findings include other substances than creatine; (c) the possibility that in muscular dystrophy guanidine intoxication plays a prominent rôle.

The present paper deals primarily with the guanidine question with some attention to the creatine to creatinine ratio. Unsubstituted guanidine was early shown by Gergens and Baumann (3) and by Putzeys and Swaen (4) to have an injurious action, especially on muscle function, findings later corroborated by Fühner (5); while Kawakita (6) found guanidine toxic for plants, a finding verified by unpublished work from this laboratory.

To facilitate the study of guanidine in relation to health and disease Sullivan (7) devised a new test with a high degree of specificity for unsubstituted guanidine,  $\text{NH}\cdot\text{C}(\text{NH}_2)_2$ . This test is not given by any substituted guanidine. Accordingly, it is not given by methylguanidine which might be formed from creatine and creatinine by oxidation, as early shown by Dessaignes (8) and corroborated by Ewins (9), Baumann and Ingvaldsen (10), and by Greenwald (11).

When the guanidine reaction was applied to urines, little evidence was obtained of the presence of unsubstituted guanidine in normal or pathological urine. A search was then made for simple derivatives comparable to glycocyamine or glycocyamidine, which

might be converted readily into unsubstituted guanidine in the same manner that Dessaignes (8) and Ewins (9) formed methylguanidine from creatine and creatinine by the use of yellow oxide of mercury or silver oxide respectively.

The mercury oxide, as used by Dessaignes, was first tried but was found to have some disadvantages. In some urines the mercury sulfide was colloidal and hard to get rid of by filtering. The greatest fault is the presence of considerable ammonium salts in the final concentrate, which precipitate on adding picric acid and interfere with the determination of guanidine. Accordingly, most of the search for guanidine in urines was by means of alkaline silver oxide.

*Liberation of Guanidine*—To 150 cc. of urine, from each patient with muscular dystrophy, a 25 per cent solution of silver nitrate was slowly added, with stirring, as long as a precipitate formed and then in slight excess. Without filtering, the mixture was brought to about pH 8 by means of hot saturated barium hydroxide and set aside for 15 hours. The resulting precipitate, centrifuged and washed with water, was suspended in 100 cc. of water and freed from silver by means of  $\text{H}_2\text{S}$ . The filtrate from  $\text{Ag}_2\text{S}$  was freed from  $\text{H}_2\text{S}$  by a current of air and from barium by  $\text{H}_2\text{SO}_4$ . The resulting filtrate was concentrated on the water bath to 15 to 20 cc. and the concentrate was brought to about pH 7.5 (greenish blue to brom-thymol blue). After filtering, the solution was treated with an equal volume of a saturated solution of picric acid. In urine from patients with muscular dystrophy a precipitate speedily formed. This precipitate gave the guanidine color reaction. Recrystallized, it gave typical crystals of guanidine picrate with a melting point on quick heating of  $314^\circ$  uncorrected,  $328^\circ$  corrected, Anschutz  $328\text{--}329^\circ$ .

With this manipulation, guanidine picrate, identified by color reaction, crystalline shape, and melting point of the picrate, was obtained from four patients with progressive muscular dystrophy and in large amounts from four with pseudohypertrophic muscular dystrophy. Two patients with progressive muscular dystrophy, patients Mn and Be, and two with the pseudohypertrophic type, McC and Cy, gave enough picrate to be recrystallized several times for analysis. Recrystallized four times from water and washed with ether the picrates gave practically the theoretical N

for guanidine picrate, patient Mn 28.9, Be 28.9, McC 29.0, and Cy 29.14 (six recrystallizations); theory for guanidine picrate, 29.17 per cent N.

In two cases of progressive muscular dystrophy and one of pseudohypertrophic muscular dystrophy no guanidine could be found. No guanidine could be obtained from the urine of three cases diagnosed as myasthenia gravis, nor from one case of leucemia, one of brain abscess, five cases of cancer, and upwards of ten normal persons. Likewise, no guanidine was found in the urine of a parathyroidectomized dog furnished us by Everette I. Evans of the Bureau of Dairy Industry, United States Department of Agriculture.

The treatment with silver nitrate and barium hydroxide which yielded unsubstituted guanidine in the case of muscular dystrophy did not yield guanidine with creatine, creatinine, or arginine. Glycocyamine and glycoyamidine on the other hand readily yielded guanidine. Since only a small amount of guanidine was obtained from 100 mg. of guanine, the latter is ruled out as a mother substance of the guanidine found by us.

The liberation of guanidine from the simple guanidine complex in the urine is probably not quantitative, since glycocyamine put through the silver nitrate-barium hydroxide treatment rarely gives quantitative results. Our findings vary from 40 to 100 per cent of the theoretical guanidine and generally are about 60 per cent of the theoretical.

Despite the incompleteness of the conversion of glycocyamine to guanidine and in an analogous way the incompleteness of the conversion of the simple guanidine derivative in the urine to unsubstituted guanidine, we obtained guanidine from eight of the eleven patients with muscular dystrophy studied in this laboratory. From Cy 700 mg. of purified guanidine picrate were obtained from 1346 cc. of urine, the 24 hour amount. It would seem then as suggested by us earlier (12, 13) that the urine in muscular dystrophy contains a simple guanidine derivative or mother substance comparable to glycocyamine which yields guanidine on oxidation with silver nitrate and barium hydroxide. Weber (14) more recently has reported that glycocyamine is present in the urine in cases of muscular dystrophy.

Of the negative cases, patient Lock had been helpless for a

number of years and recently died from pneumonia. Another patient (Fr) with the typical high creatine is a spinal type and may have a spinal muscular atrophy distinct from muscular dystrophy. The third negative case with no guanidine in the urine has been diagnosed as pseudohypertrophic muscular dystrophy. This patient, a boy 17 years old, had been under treatment with ephedrine and glycine for some time before coming to us for study. It can be said, however, that by the procedure given in this paper we have isolated guanidine as a picrate from the urine of several individuals given 10 to 15 gm. of glycine per day for many months,

TABLE I

*Presence of Simple Guanidine Derivatives in Urine in Muscular Dystrophy*

Patient	Age	Diagnosis, muscular dystrophy	Volume	Creatinine	Creatine	Creatine Creatinine	Guanidine obtained
	yrs.		cc.	mg.	mg.		
Mn	7	Progressive	340	216	305	1.4	+
Be	7	"	360	171	358	2.1	+
Bt	12	Pseudohypertrophic	585	129	174	1.35	+
Cy*	13	"	620	135	643	4.8	+
Fr	13	Progressive (spinal atrophy?)	720	466	762	1.64	-
McC	12	Pseudohypertrophic	1285	754	1014	1.34	+
Lock*	10	Progressive	435	318	928	2.9	-
Ser	14	"	510	153	366	2.4	+
Kr	7	"	960	288	674	2.34	+
Alr	10	Pseudohypertrophic	860	363	690	1.90	+
Pas	17	"	1310	947	1201	1.27	-

\* Died.

so that, if medication has altered the guanidine picture, it must be due to the ephedrine or other factors.

The chemical data covering our first analysis in each patient are given in Table I. 70 per cent of our patients diagnosed by the medical profession as cases of muscular dystrophy have given guanidine by our procedure. No evidence for the presence of simple non-methylated guanidine derivatives was obtained in three cases of myasthenia gravis. Normal urines have been invariably negative. It may be said further that our work leaves the question of the presence of methylated guanidines untouched.

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## THE CHOLESTEROL CONTENT OF MUSCLE

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Relatively little work has been done on the cholesterol content of muscle and still less attempt has been made to differentiate between different muscles or groups of muscles. In general, it has been found that the cholesterol content of muscle is lower than that of any other tissue or of blood (1). The cholesterol content of the heart has been found to be the highest, generally 2 or 3 times that of skeletal muscle (2-5). Cholesterol was found to be higher in calf muscle than in the same muscles in the cow (6). In beriberi Lawaczek (3) reported that there were definitely high values for cholesterol in skeletal muscle but not in heart muscle. Removal of the thyroid gland, according to Onizawa (7), produced a lowering of cholesterol in skeletal muscle, while Pasternak and Page (8) found that thyroxine, although it markedly increased the phospholipid content of muscle, had no effect on the cholesterol content.

In a previous communication (5), it was shown that the phospholipid content of a muscle was related to its activity, *i.e.* the amount of work which it habitually did. The greater the amount of work, the higher was found to be the phospholipid content. The cholesterol content varied in the same direction but generally not to the same extent, so that the phospholipid to cholesterol ratio was generally higher in the more used muscle. Smooth muscle was found to have a higher cholesterol content than either skeletal or heart muscle and since its phospholipid content was moderate or low, the phospholipid to cholesterol ratio was always low. The work has since been extended to include a greater number of muscles in a wide variety of species, as the result of which definite regularities have been found which will be reported and discussed in this paper.

The analytical methods used were the same as those employed in the earlier work (5). The muscle after being freed from visible fat, nerve, and connective tissue was finely ground with sand. The lipids were extracted from the finely ground tissue by boiling with alcohol followed by peroxide-free ether, the final mixture consisting of about 3 parts of alcohol to 1 of ether. After making up to suitable volume, an aliquot containing 4 to 6 mg. of phospholipid and 0.5 mg. of cholesterol was measured out, the solvent removed, and the residue rectified by extraction with low boiling (60°) petroleum ether. After removal of most of the petroleum ether, the phospholipids were precipitated by excess of acetone together with a minimum of magnesium chloride to insure complete precipitation. The precipitated phospholipid was determined by oxidative measurement.

The clear supernatant acetone solution containing the cholesterol was evaporated to dryness, the residue dissolved in chloroform, and the cholesterol determined colorimetrically by the Liebermann-Burchard reaction carried out at 20–22°, with special precautions to avoid contamination of the reaction mixture with water or alcohol. In the case of smooth muscle, the aliquot taken to contain the desired amount of phospholipid generally contained 2 or 3 times too much cholesterol for the standard used (0.5 mg.), so that it was necessary to take an aliquot of the chloroform extract containing the cholesterol for the determination.

In most cases, also, phospholipid fatty acids were determined and these values served as a check on the direct determinations of phospholipid, since the muscle phospholipid has always been found to yield 65 to 70 per cent of fatty acid.

Muscles or groups of muscles were selected as follows: muscles of the jaw, neck, upper arm and forearm, back (outer aspect of back-bone), and loin (inner aspect of back-bone), abdominal wall, diaphragm, thigh, tail, gastrocnemius, and pectoral muscles; of smooth muscles, those of the stomach, intestine, and uterus of mammals and of the gizzard of birds. The muscles of the grasshopper used were the mass of muscles in the thorax and those of the thigh. Of heart muscle, the ventricle muscle was ordinarily used. When the heart was large enough, separate analyses were made of left and right ventricles and occasionally of the auricles.

Except in the very smallest animals (mouse, bat, sparrow, and grasshopper), selections of eight to twelve muscles or muscle groups were analyzed in each animal.

As found in the earlier work on phospholipid in muscles (5), the muscles group themselves according to cholesterol content into the following classes: (a) most of the skeletal muscles, of which the thigh is taken as typical; (b) a certain group of the skeletal muscles, including the neck, diaphragm, abdominal wall, and jaw muscles, which are in more constant use and have constantly a somewhat higher lipid level; (c) heart; and (d) smooth muscles. The muscles in the skeletal groups in the same animal were found to have a considerable similarity of lipid composition and for economy of space only typical ones (thigh and diaphragm or abdominal wall) are reported (Table I). The smooth muscles are reported in detail (Table II). In addition to the cholesterol, the values of the phospholipid to cholesterol ratio,  $P/C$ , are given in both sets of muscles, since it has been found that this ratio shows differences in muscles which would not be revealed by the phospholipid or cholesterol values alone. The significant results of the analyses for cholesterol and the phospholipid to cholesterol ratio are summarized in Table III.

#### *Observations*

*Cholesterol Content*—There is a wide range of cholesterol content in each group of muscles both between species and between individuals of the same species. It cannot be said, therefore, that muscle of any kind has a closely constant and characteristic cholesterol content. On the other hand, the three conventional types of muscle (striated, cardiac, and smooth) are quite sharply differentiated from each other by their cholesterol content, smooth muscle having the highest, cardiac muscle next highest, and striated muscle the lowest content. A less marked differentiation may be noted between the main group of striated muscles and a "semivital" group, including those muscles which are in relatively continuous use—the diaphragm, abdominal wall (especially in birds), neck, and jaw muscles—and which have a higher cholesterol content than the other skeletal muscles. Among the cardiac muscles there appears to be a definite progressive increase of cholesterol values from the mammals to the birds and to the cold

TABLE I  
Cholesterol of Muscles (Per Cent of Dry Weight)

Animal	No. of animals	Heart (ventricle)			Skeletal (thigh)			Diaphragm		
		Cholesterol		Phospholipid Cholesterol	Cholesterol		Phospholipid Cholesterol	Cholesterol		
		Average	Range		Average	Range		Average	Range	
Mammals										
Man.....	5	0.70		10				0.37		
Sea-lion.....	1	0.40		14						
Kangaroo.....	1	0.32		21						
Dog.....	3	0.61	0.49-0.90	14	0.17	0.37-0.48	14	0.37	0.31-0.38	16
Cat.....	5	0.44	0.34-0.50	13	0.32	0.12-0.28	25	0.35	0.20-0.27	16
Laboratory rabbit.....	8	0.57	0.40-0.83	16	0.19	0.09-0.25	10	0.24	0.33-0.60	11
Wild rabbit.....	7	0.45	0.36-0.60	17	0.17	0.17-0.33	15	0.47	0.20-0.36	13
Jack-rabbit.....	1	0.38		19	0.25	0.17-0.33	25	0.29	0.13-0.38	14
Guinea pig.....	5	0.44	0.31-0.53	13	0.35	0.23-0.38	10	0.32	0.26-0.60	15
Gopher.....	1	0.80		12	0.33	0.21-0.36	14	0.32		
Rat.....	10	0.53	0.34-0.62	15	0.25	0.34-0.54	16			
Mouse.....	4				0.42					
Average.....		0.51		15	0.27		17	0.34		14
Birds										
Wild duck.....	1	0.52		15	0.21	0.13-0.30	18	0.40	*	8
Hen.....	10	0.54	0.41-0.70	14	0.25	0.30-0.55	15	0.34	0.29-0.44*	10
Pigeon.....	12	0.52	0.29-0.59	14	0.41	0.30-0.55	11	0.47	0.40-0.50*	11
Sparrow.....	4	0.57		15	0.40	0.27-0.30	13	0.45	0.38-0.52*	8
Owl.....	3	0.56	0.52-0.64	11	0.28		14	0.42		9
Average.....		0.54		14	0.31					

Cold blooded												
Turtle.....	6	0.80	0.75-0.90	8	0.35	0.25-0.50	10	0.28	*	14		
Frog.....	8	0.70	0.62-0.76	6	0.20	0.17-0.29	19					
Alligator.....	2	0.75		10	0.17		12					
Grasshopper.....	4				0.18		30					
Average.....		0.75		8	0.23		18					
Special												
Turtle auricle.....		1.5		3.5								
Alligator auricle.....		1.8		4			14					
Bat pectoralis.....					0.57							
New born wild rabbit (thigh).....					0.75		9					

The scientific names of the animals used other than the common laboratory animals are as follows: sea-lion *Zalophus californianus*, kangaroo *Macropus rufus*, wild rabbit *Sylvilagus floridanus*, jack-rabbit *Lepus townsendii*, gopher *Citellus richardsonii*, mouse *Peromyscus maniculatus*, wild duck *Colymbus septentrionalis*, sparrow *Passer domesticus*, owl *Scops asio*, turtle *Pseudemys elegans*, frog *Rana pipiens*, alligator *Alligator mississippiensis*, grasshopper *Brachystola magna*, bat *Myotis lucifugus*.

\* Abdominal wall.

blooded animals. These values approach and, in the case of auricle muscle in cold blooded animals, surpass the values of smooth muscle.

Occasional unexplained high cholesterol values are noted, as, for example, in the cardiac muscle of man and gopher and of the

TABLE II  
*Cholesterol of Smooth Muscle (Per Cent of Dry Weight)*

Muscle	No. of animals	Cholesterol		Phospholipid Cholesterol
		Average	Range	
Stomach (wild rabbit).....	4	0.50	0.45-0.65	5
Intestine (cat).....	5	0.81	0.70-0.90	4
Gizzard (hen).....	9	0.50	0.34-0.64	4
" (pigeon).....	12	0.63	0.55-0.74	4
" (owl).....	1	1.00		3.5
" (sparrow).....	1	0.72		4
Uterus (human).....	1	1.00		3.5
" (laboratory rabbit).....	18	1.10	0.72-1.30	4
" (dog).....	1	1.00		3
Average of all smooth muscle.....		0.77		4

TABLE III  
*Summary of Cholesterol Content of Muscles (Per Cent of Dry Weight)*

Muscle	Cholesterol	Phospholipid Cholesterol
Skeletal muscle.....	0.27	16
"    " (vital).....	0.34	13
Ventricle (warm blooded animal).....	0.55	14
" (cold " " ).....	0.77	8
Auricles of turtle and alligator. ....	1.65	3.7
Smooth muscle (gastrointestinal tract) .....	0.70	4
"    " (uterus).....	1.05	3.5

cold blooded animals. These high values are not balanced by a correspondingly high phospholipid content so that the phospholipid to cholesterol ratio is low. High cholesterol values are also found in the thigh muscles of mouse, pigeon, and sparrow. In the mouse muscle the phospholipid and cholesterol are both

high so that the  $P/C$  ratio is average, while in the pigeon and sparrow the  $P/C$  ratio is low, owing to a relatively high cholesterol value. Low cholesterol values within the normal range are frequently found among the skeletal muscles but these are generally balanced by a low phospholipid value so that the  $P/C$  ratio is not outside the normal range.

*Differences in Cholesterol Content in Different Skeletal Muscles of Same Animal*—Lawaczek (3) found the following differences in the cholesterol content in the skeletal muscles of the cock: breast 0.069 per cent (moist weight), thigh 0.106 per cent. Embden and Lawaczek (9) found, in rabbit muscles, the following percentages of cholesterol in terms of moist tissue: semitendinosus 0.079, biceps femoris 0.051, and diaphragm 0.071.

Our own results, in those cases in which we had data enough on one species for comparison, showed that in most animals, *i.e.* the dog, cat, wild rabbit, guinea pig, white rat, turtle, and frog, with few exceptions, the cholesterol content of the various skeletal muscles was much the same in each individual. In the laboratory rabbit, the values in the thigh (0.17 per cent) were lower than the average for the rest of the muscles. In the hen and pigeon, the pectoralis muscles had a notably lower (about half) value than the other skeletal muscles. From this it appears to be the rule that the cholesterol content of different skeletal muscles in the same animal tends to be the same.

*Phospholipid to Cholesterol Ratio*—The values of this ratio are the same for most cardiac and skeletal muscles, averaging about 14 and ranging between 10 and 20. For smooth muscles, the ratio is much lower, ranging between 3 and 5 and averaging 4, the low value of the ratio being due to the high cholesterol, since the phospholipid content may be the same as that of skeletal muscle. The notable exceptions to the general rule of high phospholipid to cholesterol ratio in cardiac and skeletal muscle are (a) the low ratios in cardiac (ventricle) muscle of the cold blooded animals ( $P/C = 8$ ) and in the abdominal wall in birds ( $P/C = 9$ ), which approach the values for smooth muscle, and especially (b) the cholesterol and  $P/C$  ratio values in the auricle muscle of cold blooded animals, both of which equal the values found in smooth muscle.



## DISCUSSION

The outstanding features of the present work are the definite differences in the cholesterol content of the three conventional groups of muscles, the striated or voluntary muscles having the lowest cholesterol content, the cardiac (ventricle) muscle next, and the smooth muscle the highest. The auricle muscle of the cold blooded animals falls within the smooth group. The difference in cholesterol content is widest between the characteristically voluntary muscle of the limbs and the characteristically involuntary smooth muscles, the heart muscle coming between. Shadings from these clear cut differences are noted in the continuously used "semivital" muscles, the diaphragm, jaw, neck, and abdominal wall, which have a higher cholesterol content and a somewhat lower  $P/C$  ratio than the remainder of the skeletal muscles; also in the ventricle muscles of the cold blooded animals, which differ from those of warm blooded animals in higher cholesterol values and lower  $P/C$  ratios. They tend in composition in the direction of smooth muscle. A still further tendency toward the composition of smooth muscle is shown in the auricle muscles of the cold blooded animals, which have the same cholesterol and  $P/C$  values as smooth muscle. (The few auricle muscles of warm blooded animals examined are within normal ranges of variation for ventricle muscle.)

The characteristic physiological properties of the three types of muscle are as follows (10):

*Voluntary muscle*, ability to contract quickly and strongly and to be tetanized, but not spontaneously active; capable of large expenditures of energy; single nerve supply, which is from the outside.

*Cardiac*, spontaneous rhythmic activity; absolute refractory period, so that tetany is impossible; "all or none" contraction; intrinsic nerve supply and regulatory nerve supply from the outside.

*Smooth*, spontaneous rhythmic activity, ordinarily with small tensions and slow motions requiring relatively small energy expenditure; intrinsic nerve net which insures the spontaneous activity; regulatory nerve supply from the outside.

Shadings in properties and behavior between these types are well recognized by muscle physiologists, so that differences in chemical composition are to be expected.

The most outstanding differences in behavior between the groups of muscle are, then, that smooth and cardiac muscles are spontaneously active and have a more varied and probably quantitatively greater nerve supply than skeletal muscle; cardiac and skeletal muscle have habitually a much greater energy expenditure than smooth muscle; skeletal muscle is not spontaneously active and has the least varied nerve supply. Associated with their spontaneous activity and more extensive nervous mechanism, smooth and cardiac muscles have a much higher cholesterol content than skeletal muscle, while skeletal and cardiac muscles, with their greater energy production, have a higher phospholipid content.

It seems probable that these associations represent, to some extent at least, real functional relations, that cholesterol is related to spontaneous activity of smooth and cardiac muscle, and phospholipid to energy expenditure.

Smooth muscle has a  $P/C$  ratio (3:4) which is of the same order of magnitude as that of nerve tissue (2:3), from which it might be inferred that much of the cholesterol of smooth muscle originates in its nerve supply. A similar origin might be inferred for part of the cholesterol of cardiac and skeletal muscle; but while there is no doubt that some of the cholesterol of all muscle originates in its nerve supply, there is at present no way of determining how much has this origin and how much comes from other structures in muscle.

#### SUMMARY

The three conventional types of muscle show definite differences in their cholesterol content. Smooth muscle has the highest content, averaging 0.75 per cent of its dry weight, cardiac muscle the next with 0.55 per cent, and skeletal the lowest with about 0.3 per cent. Wide variations in cholesterol content are observed in each type, so that there is considerable overlapping of values.

Different skeletal muscles in the same animal have a similar cholesterol content.

The values for the phospholipid to cholesterol ratio are high (average 15) for skeletal and cardiac (ventricle) muscles and low for smooth muscle (average 4).

The significance of the different cholesterol levels in relation to nerve supply and automaticity is discussed.

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## STUDIES ON OXIDATION-REDUCTION

### XXII. LAPACHOL, LOMATIOL, AND RELATED COMPOUNDS

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Hydroxynaphthoquinones have been isolated from plant materials of wide variety. Reference to several of these quinones was made in a previous paper (1); among them were lapachol and lomatiol. Lapachol is a yellow pigment present in the wood of many trees (3); lomatiol is obtained from the seeds of *Lomatium ilicifolia* (9). Since these two compounds are the oxidants of reversible oxidation-reduction systems, information concerning their potentials is valuable in defining the intensity level of energy changes in the environment native to these compounds. Data are here presented on the oxidation-reduction potentials of these systems over a wide pH range. Four other systems, whose oxidants are synthetic compounds structurally related to lapachol and lomatiol, are included in the study. These are included for the purpose of obtaining information concerning the relationship of chemical structure to free energy changes.

#### Results

The compounds used in this study were generously donated by the late Dr. S. C. Hooker. With the exception of lomatiol, they were all employed without further purification. The sample of lomatiol was recrystallized from benzene upon the suggestion of Dr. Hooker. All of the compounds are weak acids with a pK value of approximately 5.0; in each case the undissociated form has a pale yellow color, the anion a dark red. The reductants are colorless.

The procedure for potentiometric measurement was similar to that described for stable systems in previous papers of this series.

It was necessary to substitute veronal buffers as described by Michaelis (7) for the borate system, since borates cannot be used with polyhydroxy compounds. In order to obtain the reductants a 0.0005 M solution of the sodium salt of each compound was treated with hydrogen in the presence of a platinized asbestos catalyst. The undissociated compounds cannot be used for this purpose, since they are sparingly soluble in water. Reduction

TABLE I  
*Relation of  $E'_0$  to pH*

Temperature 30.0°.

pH	Titration agent	Lapachol	Lomatol	Hydroxy- hydro- lapachol	Hydro- lomatol	Hydroiso- lomatol	Di- hydroxy- hydro- lapachol
1.087	Mixture	+0.2345	+0.2327	+0.2324	+0.2278	+0.2375	+0.2405
2.128	"	+0.1750		+0.1719	+0.1677	+0.1778	+0.1808
2.134	$K_3Fe(CN)_6$		+0.1714				
2.967	Mixture	+0.1207	+0.1210				
4.085	"	+0.0529	+0.0515				
4.644	"	+0.0199		+0.0146	+0.0109	+0.0192	+0.0204
4.645	$K_3Fe(CN)_6$	+0.0180	+0.0155				
5.228	Mixture	-0.0258	-0.0242				
5.826	"	-0.0756	-0.0837	-0.0782	-0.0805	-0.0762	-0.0757
6.489	"	-0.1342	-0.1384	-0.1363	-0.1385	-0.1335	-0.1334
7.320	$K_3Fe(CN)_6$	-0.2090	-0.2122	-0.2099	-0.2103	-0.2083	-0.2083
7.320	Quinone	-0.2093					
7.320	$Na_2S_2O_4$	-0.2092					
8.318	Mixture	-0.2968	-0.2985	-0.2955	-0.2977	-0.2938	-0.2932
9.021	"	-0.3478	-0.3479				
10.627	"	-0.4517	-0.4540	-0.4510	-0.4557	-0.4424	-0.4373
11.006	"					-0.4602	-0.4547
11.014	$K_3Fe(CN)_6$	-0.4760	-0.4761				
11.352	Mixture	-0.4920	-0.4928	-0.4909	-0.4926	-0.4733	-0.4669
12.006	"	-0.5261	-0.5249			-0.5032	-0.4916
12.625	"	-0.5470	-0.5460	-0.5433	-0.5504	-0.5229	-0.5125

proceeded rapidly in contrast to the slow reduction observed by Fieser (4) for some of these compounds in acid alcoholic solutions. All potentials here recorded are at 30° and have been brought to the hydrogen standard in accordance with the conventions of Clark (2). Corrections were made for change of potential attributable to those changes of pH which are caused by the products formed in the oxidation-reduction process.

The data obtained are summarized in Table I. The final concentration of pigment was approximately 0.0001 M throughout. In the case of lapachol and lomatiol, titrations were performed at various pH values and in no case were there any indications that

TABLE II

*Normal Potential and Dissociation Exponents of Various 2-Alkyl-3-Hydroxy-1,4-Naphthoquinones*

Temperature 30.0°.

Alkyl group	$E_0$	$pK_0$	$pK_{r_1}$	$pK_{r_2}$
$\begin{array}{c} \text{CH}_3 \\   \\ -\text{CH}_2 \cdot \text{CH} = \text{C} \\   \\ \text{CH}_3 \end{array}$ Lapachol	0.3000	5.02	8.74	11.84
$\begin{array}{c} \text{CH}_2\text{OH} \\   \\ -\text{CH}_2 \cdot \text{CH} = \text{C} \\   \\ \text{CH}_3 \end{array}$ Lomatiol	0.3000	4.88	8.67	11.70
$\begin{array}{c} \text{CH}_3 \\   \\ -\text{CH}_2 \cdot \text{CH}_2 \cdot \text{C} \\   \quad   \\ \text{OH} \quad \text{CH}_3 \end{array}$ Hydroxyhydrolapachol	0.3000	4.94	8.63	11.72
$\begin{array}{c} \text{CH}_3 \\   \\ -\text{CH}_2 \cdot \text{CH}_2 \cdot \text{C} \\   \quad   \\ \text{H} \quad \text{CH}_2\text{OH} \end{array}$ Hydrolomatiol	0.2955	5.02	8.74	11.75
$\begin{array}{c} \text{CH}_3 \\   \\ -\text{CH}_2 \cdot \text{CHOH} \cdot \text{C} \\   \quad   \\ \text{H} \quad \text{CH}_3 \end{array}$ Hydroisolomatiol	0.3055	4.77	8.32	11.40
$\begin{array}{c} \text{CH}_3 \\   \\ -\text{CH}_2 \cdot \text{CHOH} \cdot \text{C} \\   \quad   \\ \text{OH} \quad \text{CH}_3 \end{array}$ Dihydroxyhydrolapachol	0.3080	4.76	8.30	11.08
$-\text{CH}_3$ Phthiocol	0.2987	5.06	8.68	11.70

other than two equivalents were concerned in the reaction. It was therefore deemed unnecessary to titrate the other compounds at different pH values, since they are so similar in structure. It was, however, impossible to obtain titration curves of lapachol in the acid region because of the marked insolubility of the undis-

sociated form of this compound. In fact those values reported for this compound in the acid region were obtained by extrapolating to zero time the drift of potential caused by the precipitation of the oxidant when a "mixture" was added to the various buffer solutions. Data presented under the heading of "mixture" refer to the potential obtained when a solution of the reductant mixed with a deaerated solution of the original oxidant is added to a deaerated buffer solution. The percentage oxidation of the mixture was determined by its potential in a buffer solution previously used in a titration experiment.

The electrode equation, stated with numerical coefficients for 30°, that describes the behavior of all the compounds throughout the pH range studied is as follows:

$$E_A = E_0 + 0.03006 \log \frac{[S_o]}{[S_r]} + 0.03006 \log \frac{[K_{r1}K_{r2}(H^+) + K_{r1}(H^+)^2 + (H^+)^3]}{K_o + (H^+)}$$

Here  $(H^+)$  is the hydron activity,  $[S_o]$  and  $[S_r]$  the molar concentrations of total oxidant and total reductant. The values for the various constants for each compound are listed in Table II. The dissociation constants must be defined as apparent because in the derivation of the above equation we have assumed constant ionic strength and solutions in which the buffer salts predominate. The constants for phthiocol, which are included for comparison, have been revised slightly from the previously assigned values (1) as a result of the larger scale plot of the  $E'_0$ -pH curve.

The compound lapachol, hydroxyhydrolapachol, and dihydroxyhydrolapachol have been adequately described by Hooker (5). The formula for lomatiol has recently been revised by Dr. Hooker to that given here. This information, as well as the formulas for hydrolomatiol and hydroisolomatiol, was received by the author in a personal communication from Dr. Hooker. I am informed that Dr. Hooker's work describing these compounds will appear shortly (6).

#### DISCUSSION

There is little difference in the normal potentials of the various systems. Apparently changes in the size of the alkyl group or the presence or absence of a double bond in this group has little influence on the normal potential. The presence, however, of a

hydroxyl group in the side chain has a tendency to increase the normal potential slightly; the nearer the quinonoid ring the greater the effect. These facts have been pointed out previously by Fieser (4) for alcoholic solutions of a large series of such compounds. Working at 25°, he reports normal potentials for alcoholic solutions of the systems whose oxidants are lapachol, lomatiol, and hydroxyhydrolapachol of 0.287, 0.294, and 0.295 respectively. These values are in good agreement with those reported here when the difference in solvent is considered.

It was thought at the outset of this investigation that changes in the alkyl group might have more effect upon the dissociation exponents of the components of the system than upon its normal potential. Such an effect would be manifested in a change in the potential of the system in the neutral and alkaline range, especially if the oxidant was involved. However, the observed effect on the dissociation exponent of the oxidant is not striking. The substitution of a hydroxyl group in the  $\beta$  position of the side chain tends to increase the value of this exponent only slightly. The effect of this change on the potential values in the neutral range is small, since it is offset by the higher normal potential of these compounds, so that, in the region of pH 7.0, all of the systems converge and register approximately the same potential. In the case of the reductants, changes in the alkyl group are reflected more definitely in their dissociation exponents. The values for these exponents are increased apparently according to the number and nearness to the ring of the hydroxyl groups substituted in the side chain. As a result the potentials of the various systems deviate as much as 38 millivolts at pH 12.6.

Two of the compounds studied, lapachol and lomatiol, possess unsaturated groupings in the side chain. Some doubt might be expressed that such a grouping would survive the hydrogenation procedure employed in obtaining the hydroquinone. That this grouping remains unchanged by this procedure is supported by the following evidence. First, the titration of lapachol with dithionite gives values identical with those obtained by titrating the hydroquinone. Second, the values for lomatiol are not the same as those given by hydrolomatiol, the compound which would result if this change occurred. Third, Monti (8) has shown that hydrogenation of the side chain of lapachol cannot be effected by



hydrogen and palladium catalyst. Fieser (4) reports that hydrogenation occurs readily with Adams' catalyst at about 40 pounds pressure of hydrogen.

Lapachol and lomatiol are described as existing as yellow pigments in their natural environments. Since the  $pK$  value of both of these compounds is approximately 5.0, one might be led to assume that the  $pH$  of their environment was more acid than  $pH$  5.0. This need not necessarily be the case, since the yellow undissociated form is markedly insoluble in contrast to the red salt. The insoluble yellow form then could mask the color of a smaller amount of dissolved pigment. Both pigments have been described as occurring in the solid state in the material from which they were isolated. There is no indication in the literature that the reductants of these pigments occur in nature. In the case of lapachol the recording of such an observation is unlikely, since most workers have employed imported dried wood in which the reductant would have been oxidized by air. Lomatiol appears to occur on the outside coating of seeds and hence no reductant would be expected to be found there. The presence of lomatiol inside the seed is not recorded.

The relation of the various systems described here to other known systems is similar to that of the phthiocol system (1). None of these compounds presents any advantages over phthiocol as an oxidation-reduction indicator.

#### SUMMARY

The oxidation-reduction potentials of the systems whose oxidants are lapachol and lomatiol, two naturally occurring pigments, and four other closely related hydroxynaphthoquinones of synthetic origin, are recorded at  $pH$  values ranging from 1.1 to 12.6. The effect of alterations in structure on the oxidation-reduction potentials and the dissociation exponents of the various compounds is discussed.

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**THE OXIDATION OF THE AMINO ACIDS  
THE ESTIMATION OF THE KETO ACIDS AND THE PRODUCTION  
OF HYDROGEN PEROXIDE\***

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From a consideration of the amount of oxygen utilized in the oxidation of the non-natural isomers of the amino acids by a purified enzyme preparation, it seemed probable that the corresponding keto acids were formed as end-products (1). Krebs (2) has shown that keto acids are formed in the first stage of the oxidation of the amino acids and has isolated the dinitrophenylhydrazones of valine, leucine, alanine, and phenylalanine. In order to get good yields, however, he had to use arsenic-poisoned tissue slices. We have attempted to estimate quantitatively the keto acid production when a purified enzyme preparation was used which oxidized only the non-natural isomers of the amino acids. In order to do this two different methods were employed. The first was the isolation and identification of the phenylhydrazones and the second was the formation of the bisulfite compounds and their estimation by titration with iodine. The yields obtained by the two methods varied considerably, which was probably due to structural differences in the keto acids, which affected the formation of the bisulfite and phenylhydrazone compounds. In all cases one or both of the methods gave a yield of more than 50 per cent, which indicated that the keto acids formed were not further oxidized by the enzyme preparation.

During the oxidation of the non-natural isomers of the amino acids it was noticed that methemoglobin was formed. This was shown to be due to the formation of hydrogen peroxide, which

\* Part of the expense of this work was defrayed by a grant from the Duke University Research Council.

caused the oxidation of hemoglobin to methemoglobin in the presence of the enzyme preparation. That the formation of hydrogen peroxide occurs, when alanine is oxidized, has been shown by Keilin and Hartree (3).

#### EXPERIMENTAL

For the preparation of the phenylhydrazones the purified enzyme was made from the kidneys of rats by the method already described (1), with the exception that 0.5 per cent potassium acetate with a pH of approximately 8.0 was used instead of the phosphate buffer. The kidneys of ten to fifteen rats were used, depending on their size. 50 to 100 mg. of the non-natural isomer of the amino acid were oxidized at one time and the course of the oxidation was followed manometrically. When *dl* mixtures were used, the natural isomers, with the exception of proline, were not attacked by the enzyme (1). The oxidation was complete in 1 to 3 hours, depending on the amino acid. At the end of the oxidation acetic acid was added to the mixture until the protein began to flocculate and it was then heated in a boiling water bath for 5 minutes to complete the precipitation. It was filtered through muslin and then filter paper, the precipitate in the muslin being pressed as dry as possible. The clear yellow filtrate was evaporated in an air current at a temperature not over 60° to a volume of approximately 10 cc. Any flocculent precipitate that was formed during the evaporation was filtered off.

To prepare the phenylhydrazone the filtrate was made alkaline with potassium carbonate, a slight excess of phenylhydrazine hydrochloride was added, and the mixture was allowed to stand for 2 hours at room temperature. It was then acidified with hydrochloric acid and the precipitate filtered, washed, and weighed when dry. Better yields were obtained and less phenylhydrazine decomposed when potassium carbonate and hydrochloric acid were used instead of potassium acetate and acetic acid. The precipitates were crystalline and light yellow. They were then recrystallized from alcohol and their melting points determined. Recrystallization did not change the color of the compounds. The original crystals were completely soluble in 95 per cent alcohol, showing that they were free from inorganic salts.

For determining the bisulfite compounds only 1.0 mg. of amino acid was oxidized and 0.05 M phosphate buffer, pH 7.8, was used.

At the end of the oxidation the total volume of the mixture, which amounted to 2.0 cc., was poured into 1.0 cc. of 5 per cent trichloroacetic acid. The resulting mixture was then diluted to 10 cc. with water and filtered. An aliquot part, usually 5 cc. of the clear filtrate, was taken and excess bisulfite added. The procedure then followed that of Clift and Cook (4), except that the bisulfite was allowed to stand for half an hour before titrating the excess with iodine. Controls were run without bisulfite to show that neither the amino acids themselves nor their oxidation products reduced iodine in alkaline solution at room temperature. This was true in all cases except for tyrosine, methionine, and histidine. The bisulfite method was therefore inapplicable for these three amino acids because no sharp end-points could be obtained when the unoxidized acids were titrated. It was possible, however, to show qualitatively that the oxidation product of histidine formed a bisulfite compound.

The results for each amino acid are given in Table I. All melting points are uncorrected. The phenylhydrazones of the keto acids from leucine and methionine are not described in the literature. In order to establish the identity of these two compounds an iodine titration method was used. This method was suggested by the work of Humphries and Evans (10) on the bromination of phenylhydrazine derivatives. They found that 2 bromine atoms were taken up per molecule of phenylhydrazine. We used iodine instead of bromine. The phenylhydrazone was dissolved in slightly alkaline solution in a concentration of 10 mg. per 100 cc. Aliquot amounts were taken and acidified with 0.1 N HCl. In slightly acid solutions the phenylhydrazones in this concentration do not precipitate. The solution was treated with a known excess of standard iodine solution and allowed to stand in the dark for half an hour at room temperature. The excess iodine was then titrated with standard thiosulfate. In all cases 2 molecules of iodine were taken up per molecule of phenylhydrazone. The method was checked with phenylhydrazine itself and with the known phenylhydrazone of isoleucine. The 2,4-dinitrophenylhydrazone of methionine was prepared and this reduced no iodine, indicating that iodine goes into the ring in the 2,4 positions and that only the phenylhydrazine part of the molecule is attacked. The results are shown in Table II. The use of iodine as a general method for estimating phenylhydrazones is being worked out.

Free phenylpyruvic acid was isolated by extraction with ether after the oxidation of phenylalanine with a yield of 47 per cent.

TABLE I  
*Yields of Phenylhydrazone and Bisulfite Compounds Obtained From Amino Acids*

Except in the case of proline only the non-natural isomers were oxidized when the *dl* mixtures were used (1).

Amino acid	Percentage yield of bisulfite compound	Percentage yield of phenylhydrazone	Melting point, phenylhydrazone*	
			°C.	°C.
<i>dl</i> -Alanine.....	84			
<i>l</i> -Valine.....	90	38	137	129(5) 137(6)
<i>dl</i> -Serine.....	55			
<i>d</i> -Leucine.....	81	54	125	
<i>l</i> -Isoleucine.....	54	98	131	130(7) 142(8)
<i>d</i> -Phenylalanine. ....	93	49	161	161(9)
<i>dl</i> -Methionine.....		66	135	
<i>dl</i> -Proline.....	68			
<i>l</i> -Proline.....	45			
<i>d</i> -Histidine.....	60 (Approximate)			

\* The figures in parentheses represent the bibliographic references in which the corresponding melting points given are cited.

TABLE II  
*Molecular Weights of Various Phenylhydrazones As Determined by Iodine Titration Method*

	Molecular weight		
	Calculated	Found	Average
Isoleucine	220.1	222.8 210.8	216.8
Leucine	220.1	220.0 220.4	220.2
Methionine	238.1	231.0 241.2	236.1

It had a melting point of 149°. Erlenmeyer (9) gives 154°. The oxidation product of *d*-tyrosine gave no phenylhydrazone and could

not be estimated by the bisulfite method. The enzyme preparation oxidized neither the natural nor non-natural isomers of aspartic acid.

In order to decide where the oxidation takes place in the proline molecule the following facts must be taken into consideration. First, 1 atom of oxygen is taken up for every molecule of proline oxidized. Secondly, no deamination occurs. Thirdly, methylene blue is reduced by the proline-enzyme system (1). Fourthly, a keto or aldehyde group is formed, as shown by the formation of the bisulfite compound. Finally, Weil-Malherbe and Krebs (11) have shown that glutamic acid is formed and that pyrrolidone-carboxylic acid is not an intermediate product. A scheme fitting all these facts would involve the loss of 1 hydrogen atom from the nitrogen and 1 from the adjacent carbon to which the carboxy group is not attached. This would leave a double bond which might hydrolyze to give the corresponding aldehyde. The purified preparation evidently takes the oxidation no further and thus would account for the formation of the bisulfite compound. With tissue slices, however, this aldehyde could be oxidized to the acid, thus giving glutamic acid.

#### *Formation of Hydrogen Peroxide*

If hemolyzed blood is added to the purified kidney enzyme and shaken in a Warburg vessel at 37°, pH 8.0, no change in the color of the hemoglobin can be seen by the naked eye. If, however, a non-natural isomer of the amino acid is being oxidized, the red color of the hemoglobin rapidly turns to dark brown. If the mixture is then acidified to about pH 6.0 and centrifuged and the clear solution examined with a spectroscope, the characteristic band of methemoglobin is distinctly seen. The control without the amino acid shows no band or at most a very faint one. The formation of methemoglobin can be produced in the control, if small amounts of hydrogen peroxide are added. The addition of hydrogen peroxide to hemolyzed blood alone does not produce methemoglobin under the conditions of the experiment. These facts indicate that during the oxidation of the non-natural isomers of the amino acids by the kidney preparation hydrogen peroxide is formed, which by means of a factor also present in the preparation oxidizes hemoglobin to methemoglobin. This factor is



present in brain and liver and probably in other tissues, so that the formation of methemoglobin during a biological oxidation could be used as a simple test for the production of hydrogen peroxide during the process. The test could be made quantitative by an accurate measurement of the absorption bands. Experiments with brain indicate that when natural (*l*-) proline is oxidized no hydrogen peroxide is formed, whereas the same preparation oxidizing the non-natural (*d*-) proline shows hydrogen peroxide formation. Whether this distinction between the oxidation of the natural and non-natural isomers is a general one and holds for all tissues is not yet determined.

#### DISCUSSION

The failure to get 100 per cent yields by the two methods used for the isolation of the keto acids from the oxidation mixture may be partly accounted for by experimental error and the limitations of the methods. That the structure of the molecule determines the extent to which the phenylhydrazone and bisulfite compounds can be formed is best illustrated by isoleucine and leucine. Isoleucine gave a 98 per cent yield as a phenylhydrazone but the bisulfite method gave only a 54 per cent yield. For leucine the bisulfite method gave a larger yield than the phenylhydrazone. It is probable that the yields from both methods represent equilibrium values, and the extent to which the equilibrium can be pushed in the direction of the formation of the compound will in all probability depend on the structure of the rest of the molecule. But, as the yields by one method or the other definitely exceed 50 per cent, it is unlikely that end-products other than the keto acids are formed during the oxidation of the non-natural isomers when the purified enzyme preparation is used.

#### SUMMARY

1. The phenylhydrazones and bisulfite compounds of the end-products of the oxidation of the non-natural isomers of various amino acids by a purified kidney enzyme have been quantitatively estimated.

2. The results indicate that only the keto acids are formed under the conditions of the experiments. Proline apparently is an exception.

3. Hydrogen peroxide is formed during the oxidation of the non-natural isomers of the amino acids. The formation of methemoglobin from hemoglobin is useful as a test for hydrogen peroxide formed during biological oxidations.

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## NOTE ON THE ULTRA-VIOLET ABSORPTION SPECTRUM OF TYROSINE

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Soon after the publication of our paper on the absorption spectra of amino acids<sup>1</sup> Dr. W. F. Ross and Dr. G. I. Lavin of The Rockefeller Institute for Medical Research informed us that they could not confirm our results on the absorption of tyrosine. In making a number of repetitions of our work we were unable to bring out the bands at 2605, 2540, 2470, and 2415Å. However, the first three bands (see Table I<sup>1</sup>) were observed, in complete agreement with Ross and Lavin.

At the present time we are unable to account for this discrepancy. On Fig. 1 (lower half) the last four bands appear to be definitely associated with the first three. The hypothesis is advanced that the additional bands originally reported may be explained by the formation of new products by the photochemical action of ultra-violet light. We propose to test this hypothesis in the near future.

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## THE MICRODETERMINATION OF SILICON\*

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The yellow color accompanying the formation of the silicomolybdic acid complex has been made the basis for the colorimetric determination of silicon by a number of investigators, namely Schreiner (1), Lincoln and Barker (2), Atkins (3), Dienert and Wandenbulcke (4), King and Lucas (5), and Thompson and Houlton (6). When small amounts of silicon are to be determined, the yellow color of the silicomolybdic acid complex is difficult to match with standards, although Thompson and Houlton have reported the estimation of 1 part of silicon in 50,000,000. Other investigators, namely Isaacs (7), Oberhauser and Schormüller (8), King (9), Berg and Teitelbaum (10), Némec, Laník, and Kopová (11), Pincussen and Roman (12), and King and Stantial (13), have reduced the yellow silicomolybdic acid complex with various reducing agents and obtained a blue color which is more satisfactory than yellow for colorimetric analysis.

The necessity of estimating quantitatively amounts of silicon less than 0.01 mg. and the possibility of applying the photoelectric colorimeter previously described (14) to the comparison of blue colors produced by reduction of the silicomolybdic acid complex led to the development of the following method.

The intensity and quality of the blue color resulting from reduction of the silicomolybdic acid complex are determined chiefly by two factors, the reducing agent employed and the acidity at which the reduction takes place. Since the color comparisons were to be made with a photoelectric colorimeter, with a Weston photronic cell, the following characteristics were essential. A blank de-

\* Food Research Division Contribution No. 267.

termination prepared with silica-free water plus the reagents must be free from cloudiness, must give a minimum blue color, and must give a constant reading in the colorimeter for an hour or more. The blue colors developed in the standards must likewise be free from cloudiness, must attain a maximum color within a reasonable length of time, and then remain constant for an hour or more.

King and Stantial (13) employed 1,2,4-aminonaphtholsulfonic acid as a reducing agent. This agent was tried but was discarded because of the amount of color produced in blank determinations. Other reducing agents, commonly employed as photographic developers, were tried and two of them were found useful for further study. Diaminophenol, known as amidol, dissolved in a sodium sulfite solution to retard oxidation, permitted the detection of 1 part of silicon in 200,000,000 parts of water. However, the slow but continuous oxidation of diaminophenol produces a reddish purple color, introducing a continuously changing error and making quantitative estimation impossible. Where *p*-hydroxyphenylglycine was used as a reducing agent, blank determinations showed a minimum of color, the blue color in standards was very clear, and the maximum color was reached in a short time and was followed by little or no fading. *p*-Hydroxyphenylglycine used as a reducing agent permits the detection of 1 part of silicon in 100,000,000 parts of water.

The optimum acidity for the development of the yellow silicomolybdic acid complex has been shown by King and Stantial (13) to be between 0.075 and 0.085 *N* sulfuric acid. However, in order to produce a minimum color in the blank, it is best to develop the color at a slightly lower acidity. Fig. 1 summarizes the results of a number of experiments in which studies were made of the relative intensity of blue color produced by sodium sulfite, 1,2,4-aminonaphtholsulfonic acid, diaminophenol, and *p*-hydroxyphenylglycine in the presence of varying concentrations of sulfuric acid.

In the procedure for the determination of silicon as finally established the following reagents are employed.

1. Ammonium molybdate  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$  2.5 per cent in 0.1 *N*  $\text{H}_2\text{SO}_4$ . This reagent must be prepared fresh each day and must be silicon-free, as indicated by the absence of yellow color when a few gm. are dissolved in a small amount of water, followed by the addition of sulfuric acid.

2. *p*-Hydroxyphenylglycine solution. 0.05 per cent in 2.5 per cent sodium sulfite, prepared fresh each day.

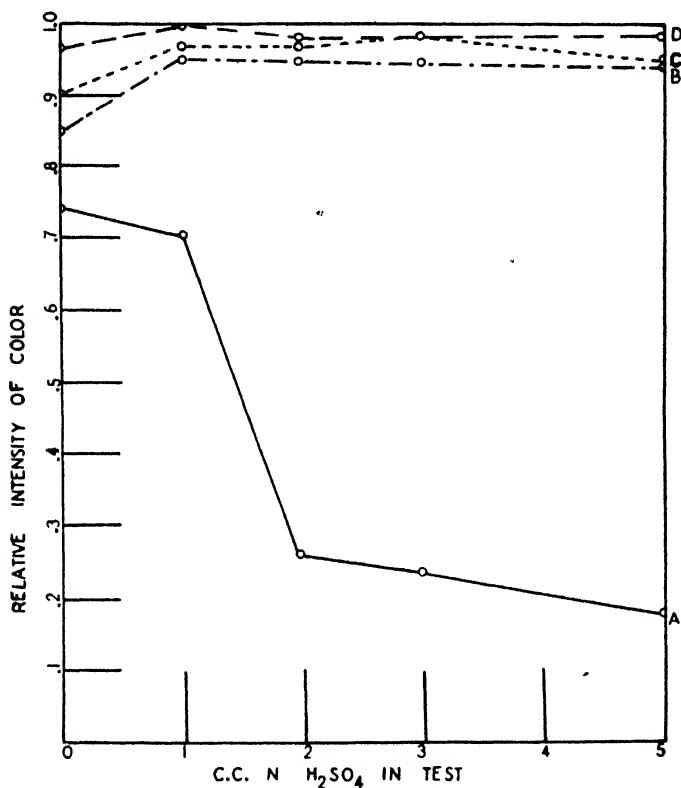


FIG. 1. Relative intensity of color in tests containing 0.02 mg. of Si, 1 cc. of 5 per cent ammonium molybdate in 0.2 N H<sub>2</sub>SO<sub>4</sub>, and various amounts of N H<sub>2</sub>SO<sub>4</sub> in a volume of 50 cc. with: Curve A, 1 cc. of 20 per cent Na<sub>2</sub>SO<sub>3</sub> (color allowed to develop 30 minutes at room temperature); Curve B, 1 cc. of 5 per cent diaminophenol in 3 per cent Na<sub>2</sub>HSO<sub>3</sub> (color allowed to develop 30 minutes at room temperature); Curve C, 0.5 cc. of 0.2 per cent 1,2,4-aminonaphtholsulfonic acid (color allowed to develop 10 minutes at room temperature); Curve D, 2 cc. of 0.5 per cent *p*-hydroxyphenylglycine in 2.5 per cent Na<sub>2</sub>SO<sub>3</sub> (color allowed to develop 30 minutes at room temperature).

3. Silicon standard. Three separate silicon standards were prepared; one from c.p. sodium silicate Na<sub>2</sub>SiO<sub>3</sub>·9H<sub>2</sub>O, one from silicon tetrachloride, and one from c.p. sodium fluosilicate Na<sub>2</sub>SiF<sub>6</sub>.



These were standardized against pure vacuum-dried picric acid (5). Each solution was then adjusted so that 1 cc. contained 2 mg. of silicon. The stock solution was diluted as required to give a standard containing 0.01 mg. of silicon per cc.

The colors for comparison in the photoelectric colorimeter (14) are prepared in Pyrex test-tubes, the total volume being about 40 cc. To 5 cc. of a solution containing 0.001 to 0.01 mg. of silicon, 4 cc. of the ammonium molybdate reagent are added, mixed

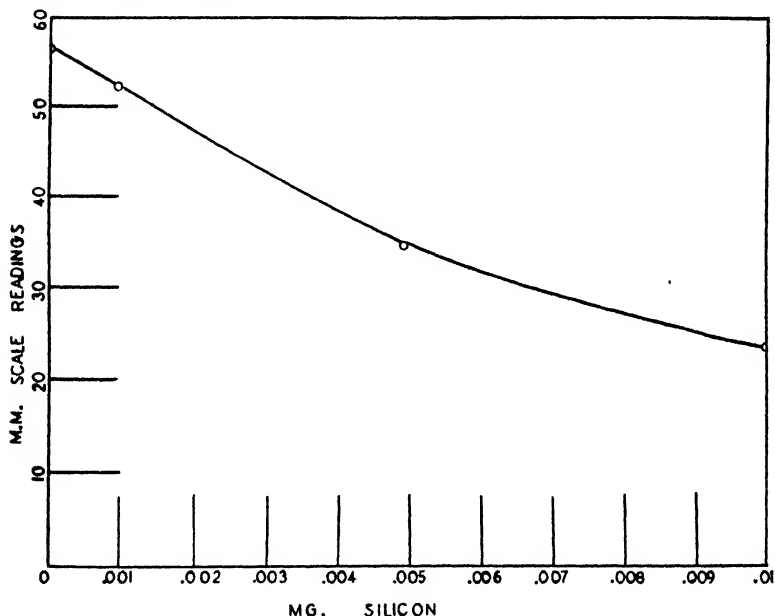


FIG. 2. Calibration curve for silicon

thoroughly, and allowed to stand for 10 minutes for the development of the yellow silicomolybdic acid complex. About 30 cc. of silicon-free water are added and thoroughly mixed. With constant stirring 2 cc. of the *p*-hydroxyphenylglycine solution are added. 30 minutes are allowed for maximum color development. The contents of the tube are then transferred to a standardized 50 cc. Nessler tube, made up to the 50 cc. mark, and the reading is taken on the galvanometer of the photoelectric colorimeter. From the position of the galvanometer reading on a calibration curve the quantity of silicon may be read off.

If the colors are always developed in precisely the same manner, it is possible to determine a calibration curve for a given set of reagents and a given Nessler tube. The calibration curve for silicon shown in Fig. 2 was determined in the following manner.

To each of four sets of four tubes designated Tubes A, B, C, and D, quantities of silicon standard were added, 0.0 cc. to Tube A, 0.1 cc. to Tube B, 0.5 cc. to Tube C, and 1 cc. to Tube D. The colors were developed in the manner described above, and the readings taken on the photoelectric colorimeter. The data given in Table I list the mean of the galvanometer readings, the probable error, and the coefficient of variation for a typical calibration curve.

TABLE I  
*Reproducibility of Calibration Curve*

	Tube A Blank	Tube B 0.001 mg. Si	Tube C 0.005 mg. Si	Tube D 0.01 mg. Si
	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>
	56.8	52.5	34.0	23.0
	56.5	52.0	34.2	22.8
	57.0	52.2	34.3	23.0
	56.5	51.8	34.2	23.5
Mean . . . . .	56.70	52.13	34.18	23.08
P.E. . . . .	0.16	0.18	0.21	0.15
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Coefficient of variation . . . . .	0.41	0.50	0.91	0.95

As stated in a previous paper (14) the character of the calibration curve is determined by the set of reagents used, the particular Nessler tube, and the particular photoelectric cell used in the photoelectric colorimeter. Changing any one of these factors necessitates the determination of a new calibration curve.

Of the various substances capable of causing interference, tungsten and arsenic are not usually found in animal tissues in troublesome amounts, but may be encountered in various soils. Arsenic in quantities less than 1.4 parts per million shows no interference, while tungsten as  $\text{WO}_4$  interferes when present in amounts greater than 8 parts per million. Aluminum in quantities above 1 part per million increases the color, while iron shows no interference in

concentration up to 10 parts per million. Phosphates increase the color when present in amounts greater than 0.2 part per million. For the determination of silicon in animal tissues both iron and phosphorus can be removed with excellent results by using the method of King and Stantial (13).

#### SUMMARY

By using *p*-hydroxyphenylglycine as a reducing agent, under appropriate conditions of concentration and acidity, for the production of a blue color from the silicomolybdic acid complex, and making colorimetric comparisons in a photoelectric colorimeter, a method has been devised for the estimation of silicon in amounts as low as 0.001 mg.

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# THE PREVENTION OF NUTRITIONAL ENCEPHALOMALACIA IN CHICKS BY VEGETABLE OILS AND THEIR FRACTIONS

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One of the disorders occurring in young chicks upon synthetic diets is characterized by severe pathological changes in the central nervous system (1-3). This disease, for which we have suggested the term *nutritional encephalomalacia*, has no correlation with body weight, sex, or breed of chick (4) and develops only during the period of active brain growth (5). The disease is not infectious nor transmissible by inoculation.

Since the disorder was observed but rarely upon diets of natural foods, experimentation on the possible dietary factors was begun by incorporating in the synthetic diet various natural foods, such as grains, grain products, green foodstuffs, animal tissues, or animal products. The ineffectiveness of these supplements led to further experiments in which the content of mineral, fat, carbohydrate, and vitamin of the synthetic diet was varied. A high proportion of lard in the diet seemed to favor the development of the disease. When the lard was replaced by vegetable oils, these were found to provide complete protection (6).

We wish to report experiments demonstrating this protective effect of vegetable oils. It will be shown also that the preventive factor is contained in the non-saponifiable fraction.<sup>1</sup>

*Care and Examination of Animals*—White Leghorn chicks of accredited stock were obtained from Hall Brothers, Wallingford,

<sup>1</sup> A summary of the work was presented at the annual meeting of the Federation of American Societies for Experimental Biology at Washington, D. C., March 25-28, 1936 (7).

Connecticut, and were kept in electrically heated brooder batteries under uniform conditions. Experimental groups of from ten to twenty birds were given the diets usually from the day of arrival in the laboratory. All chicks were weighed twice a week. When symptoms appeared, usually during the 3rd and 4th weeks of the experiment, the chicks were chloroformed. The experiments were terminated arbitrarily after periods varying from 28 to 50 days; all surviving chicks were then killed. At autopsy, the entire brain was examined for gross lesions, and then fixed in Bouin's fluid; later, two sagittal sections through each half of the brain were studied microscopically. Blocks were taken also from viscera showing gross abnormalities. About 3 per cent of the total number of chicks died during the 1st week and were discarded without examination.

The diets were freshly prepared every week and consumed within 7 to 10 days. Additional roughage or grits were not supplied. When natural foods were incorporated in Diet 108, the amounts of casein, corn-starch, lard, and salts were altered in such a way as to maintain the original relation of protein, carbohydrate, fat, and minerals. Supplements such as fresh greens were cut or ground, mixed with a small amount of Diet 108, and fed early in the day. The main portion of Diet 108 was withheld until after the supplement had been consumed. Small amounts of substances were fed by pipette or introduced into the crop by a rubber tube.

*Symptoms*—After a short period of fairly good growth and normal behavior, symptoms indicating a nervous derangement appear, often with startling suddenness. Ataxia, coarse tremors, retraction of the head, sometimes with lateral twisting; opisthotonos; forced movements, propulsive, retropulsive, or rotational; clonic spasms of the legs, increasing incoordination ending in complete prostration, stupor, and death—these are the features most often observed. Often they seem to be aggravated by the excitement of handling. When left alone, the chicks droop, close their eyes, and remain for long periods in a fixed posture.

Even when prostration is complete, and an erect posture untenable, there is not complete paralysis of wings or legs.

Not always are there such pronounced evidences of brain injury. The experienced observer can often detect slight incoordination or tremor, and these symptoms are sometimes transient, and attended

by a temporary drop in weight, followed by recovery. Quite often it happens that no conspicuous symptoms are detected during life, but that study of the brain at autopsy will show healing lesions. Rarely, extensive acute cerebral lesions are found, which during life have excited no discoverable symptoms.

*Pathology*—A complete study of the pathology has been reported elsewhere (1, 2). Only the salient anatomical features need be mentioned here. The gross lesions are confined to the brain, and are found most often in the cerebellum, less frequently in the cerebrum, occasionally in the medulla, never in the optic lobes. The appearances vary with the extent of the lesions, and the duration of the symptoms. In early cases, the affected brain tissue is swollen, soft, wet, pale, or stippled with minute hemorrhages. Later the necrotic areas become sharply delineated, and take on a greenish or yellowish green color. If the animal survives, and healing occurs, the lesions are sunken, firm, fibrous, often with a rusty discoloration due to the formation of hemosiderin.

Microscopically, the essential change is an ischemic necrosis, due to spasm or occlusion of small arterioles or capillaries.

Although the final diagnosis in these experiments was based on microscopic study of the tissue, it is possible with experience to diagnose the disease correctly from the gross lesions. In 735 positive cases, the diagnosis was thus correctly made in 639, or 87 per cent.

#### *Incidence of Nutritional Encephalomalacia*

The diet upon which the disease was first observed, and which served as a basal ration, contained the following unpurified ingredients.

##### *Diet 108*

	per cent
Skim milk powder (Merrell-Soule).....	15
Casein (Merck's technical).....	20.5
Corn-starch.....	20
Lard.....	21
Cod liver oil (Mead Johnson).....	2
Yeast, bakers'.....	5
Salt mixture (McCollum No. 185*).....	6.5
Paper pulp (Eastman).....	10

\* McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).

TABLE I  
*Incidence of Nutritional Encephalomalacia in Chicks on Diet 108 and Modifications*

Diet	No. of chicks	No. with disease	Per cent incidence
iet 108, basal ration.....	357	223	62
ontrol natural foods			
Diet 20.....	38	0	0
“ 634.....	95	2	2
iet 108, supplemented with grains or grain products*			
Ground whole wheat, 20%.....	15	0	0
“ “ “ 20%.....	12	4	33
“ “ “ 20%.....	14	5	36
“ “ “ 20%.....	13	2	15
“ “ “ 40%.....	13	0	0
Seed wheat, 3 gm. daily per chick.....	15	0	0
Seed wheat, 3 gm. daily per chick... ..	22	9	41
Wheat sprouts, 5 gm. daily per chick.....	31	5	16
Wheat products, equivalent to 40% wheat.....	100	46	46
Yellow corn, 20%.....	16	3	19
Polished rice, 25%.....	12	10	77
“ “ 25%.....	9	2	22
iet 108, supplemented with fresh greenstuffs			
Lettuce, 6 gm. daily per chick	15	0	0
“ 10 “ “ “ “	14	3	21
“ 5 “ “ “ “	15	3	20
“ 2.5 “ “ “ “	15	4	27
Spinach, 5 “ “ “ “	14	2	14
“ 5 “ “ “ “	13	0	0
Cabbage, 5 “ “ “ “	14	8	57
Grass, 5 “ “ “ “	21	2	10
“ 2.5 “ “ “ “	20	3	15
Alfalfa leaf meal, 10%.....	15	11	73
“ “ “ 10%.....	14	6	43
iet 108, supplemented with animal tissues or products			
Meat scraps, 20%.....	15	10	67

\* Diets prepared so as to maintain constant nutritive ratio.

TABLE I—*Concluded*

Diet	No. of chicks	No. with	Per cent incidence
Diet 108, supplemented with animal tissues or products— <i>Continued</i>			
Fresh beef liver, 5 gm. daily per chick.....	15	9	60
Fresh calf brain, 5 gm. daily per chick.....	17	0	0
Fresh calf brain, 5 gm. daily per chick.....	14	10	71
Fresh calf brain, 2 gm. daily per chick.....	15	10	67
Fresh calf brain, 0.5 gm. daily per chick.....	14	9	64
Fresh egg yolk, 1 cc. daily per chick.....	15	4	27
Diet 108, variations in proportions of ingredients			
Yeast increased to 20%.....	8	8	100
Cod liver oil omitted, chicks irradiated.....	19	12	63
Cod liver oil omitted, chicks irradiated, 10% butter added...	19	12	63
Minerals increased to equal value of Diet 634.....	21	14	66
CaCO <sub>3</sub> added to improve Ca:P ratio.....	34	12	35
Skim milk powder omitted.....	41	10	24
Change in lard-corn-starch ratio			
% lard        % corn-starch			
0          53.4	15	3	20
0          53.4	14	5	36
0          53.4	13	2	15
3          45.0	10	2	20
3          45.0	12	2	17
8          40.5	11	1	9
20         21.5	12	6	50
33.7         0	15	8	53

As is shown in Table I the incidence of the disease on Diet 108 was 62 per cent. The chicks were distributed in twenty-five groups and the morbidity in individual groups ranged from 30 to 100 per cent (4). There was no seasonal influence.



*Natural Foods Diets*—The normal control chicks were kept on one of two natural foods diets. Diet 20 was made up of a mash for egg-laying hens, supplemented with skim milk powder, cod liver oil, and yeast. Diet 634, containing fewer kinds of natural foods, was described by Hogan, Hunter, and Kempster (8). The composition of Diets 20 and 634 is given in Table II.

No cases developed in thirty-eight chicks on Diet 20 (Table I); two cases occurred among the 95 chicks on Diet 634. The total incidence in the controls is thus 1.5 per cent.

TABLE II  
*Natural Foods Diets*

Ingredients	Diet 20	Diet 634
	per cent	per cent
Yellow corn-meal.....	22.2	
Whole wheat.....		55.6
Wheat bran.....	16.2	
“ middlings.....	12.2	
Ground oats.....	11.0	
Alfalfa leaf meal.....	6.0	2.5
Whole milk powder.....		8.2
Skim “ “.....	18.0	
Casein.....		12.3
Butter fat.....		4.2
Cod liver oil.....	2.0	3.0
Yeast.....	2.0	12.0
Meat scraps.....	4.0	
Bone meal.....	4.0	
CaCO <sub>3</sub> .....	1.6	1.3
NaCl.....	0.8	0.9

*Diet 108, Supplemented with Grains or Grain Products, Green Foodstuffs, Animal Tissues or Products*—It is seen in Table I that the incorporation of natural foods in Diet 108 did not give consistent or complete protection. Partial protection was obtained with grains and grain products, lettuce, spinach, grass, and egg yolk; but a repetition of the experiment often yielded inconstant results. Cabbage, alfalfa leaf meal, meat scraps, and fresh beef liver apparently exerted no beneficial effect. The observations with calf brain were unusually conflicting, since in the first trial

there was complete protection and in the second, no protection at all.

*Diet 108, Variations in Proportions of Ingredients*—An increase in the yeast content of Diet 108 up to 20 per cent did not prevent the chicks from developing the cerebellar disorder, nor was the presence of cod liver oil a determining factor. Changes in the inorganic constituents likewise failed to protect the chicks. Skim

TABLE III  
*Protective Effect of Vegetable Oils Incorporated in Diet 108*

	Vegetable oil	Lard	No. of chicks	No. with disease	Per cent incidence
	per cent	per cent			
Wesson (cottonseed).....	21	0	14	0	0
Crisco ( " hydrogenated).	21	0	14	0	0
" .....	10	0	12	0	0
" .....	5	0	12	0	0
" .....	10	11	15	0	0
" .....	5	16	16	6	38
Mazola (corn).....	21	0	14	0	0
" .....	21	0	16	0	0
Peanut.....	21	0	14	0	0
Olive.....	21	0	14	3	21
Soy bean oil .....	21	0	13	0	0
" " " .....	5	16	13	0	0
" " " .....	5	16	9	0	0
" " " .....	5	16	10	0	0
" " " .....	5	16	10	1	10
" " " .....	5	16	10	0	0
" " " .....	5	16	10	0	0
" " " .....	2	16	11	1	9
Total of chicks on 5% soy bean oil and 16% lard..			62	1	1.6

milk powder was omitted from the diet to see whether it might contain sufficient of the unknown protective substance to explain the consistent survival of a few chicks on Diet 108 in each experiment. However, the elimination of skim milk powder did not increase the incidence.

Changes in the roughage content of Diet 108 did not alter the incidence to the disease. When ground cellophane was substituted for paper pulp in the diet, eleven of fifteen chicks on the diet

developed the disease; when sand was given, five of fifteen chicks on the diet presented lesions; a reduction in the amount of paper pulp from 10 to 5 per cent was also without effect.

As is seen from Table I the incidence of the disease was found to vary with the relative amounts of lard and corn-starch in the diet. On diets with low lard content the incidence was decreased. When no lard was present, the disease still appeared, indicating that the disorder was not caused by a toxicity of the lard. On a high lard diet, in which no corn-starch was added, the incidence was not increased above that usually obtained on Diet 108.

*Protection Afforded by Certain Vegetable Oils*—Since a high proportion of lard in the diet seemed to favor the development of the disease, it seemed logical to replace lard by other fats. Table III shows that certain vegetable oils do in fact prevent the disorder. Of the oils tested, olive oil alone failed to give complete protection when the oils were substituted for lard.

Table III also shows the effect of replacing part of the lard with vegetable oils. With lard in the diet, a larger amount of the vegetable oil is required for protection. Therefore, in all subsequent tests, the lard content of the diet was kept at 16 per cent or more, in order to make the conditions of the experiment more rigid.

Soy bean oil seemed to have a greater protective effect than Crisco. Only one chick of 62 in six separate groups developed the disease on the diet containing 5 per cent oil and 16 per cent lard, an incidence comparable with that on the control diets of natural foods. For this reason soy bean oil was chosen for studies on the properties and concentration of the factor. The grade of oil used was pure virgin soy bean oil that had been neither treated, heated, nor refined.

*Effect of Autoclaving Protective Foods*—When the natural foods diets were autoclaved 6 hours at 120°, the chicks developed polyneuritis, but not encephalomalacia. Both Diets 20 and 634 were autoclaved in the dry state, and, in addition, Diet 634 was first moistened with water and then autoclaved under the same conditions. None of the twenty chicks on these autoclaved diets survived beyond 14 days. They grew very little at first and then lost weight until, at the time of death, they frequently weighed less than at hatching, although the crop and gizzard contained food. Some of the chicks were found with marked head retraction

and general prostration. The others died at night without symptoms having been observed. The chicks with symptoms did not show the forced movements or marked tremors characteristic of the chicks on Diet 108. None of these polyneuritic chicks presented gross or microscopic lesions of the cerebellum or cerebrum, such as occur in encephalomalacic disease.

When untreated yeast was added to the autoclaved diets, the chicks grew, but not so well as on the control diets. Of nineteen chicks, not one developed encephalomalacia.

Soy bean oil was autoclaved for 6 hours at 120°. A deep reddish color developed and a dark gummy material settled out. Upon filtering, a pale yellow oil was separated, which, when fed at a level of 5 per cent oil and 16 per cent lard, was found to protect twenty chicks.

*Stability of the Oil to Irradiation*—Soy bean oil was exposed in a thin layer to irradiation from a Hanovia quartz lamp for 30 minutes at a distance of 3 feet. When this treated oil was fed to ten chicks in a diet containing 5 per cent oil and 16 per cent lard, one chick developed the disease. Irradiation at this intensity therefore did not materially affect the protective influence of the oil.

*Concentration of the Protective Factor*—A 20-fold concentration of the active substance was obtained by extraction of soy bean oil with 95 per cent ethyl alcohol at room temperature. With alcohol the oil readily forms an emulsion which separates after several hours. At first the alcohol layer is deeply pigmented, but after several treatments with fresh alcohol, the extract becomes almost colorless.

After removal of the alcohol from the combined extracts by distillation under reduced pressure, a reddish brown viscous residue was obtained, in which crystals formed upon standing in the refrigerator. This residue was not very soluble in 95 per cent alcohol and contained 1.5 to 3.0 per cent of the original oil. The yield was not appreciably influenced by using large amounts of solvent, nor by increasing the number of extractions.

In the preparation of the diet for testing, about 2.5 gm. of the viscous alcoholic extract were mixed into 210 gm. of melted lard and added to the rest of the ingredients in Diet 108, so that the alcoholic extract was fed at a level of 0.25 per cent. The protective

value of extracts made from different samples of oil is shown in Table IV.

It is obvious that a high degree of protection was obtained with the alcoholic extract, comparable with that given by 5 per cent soy bean oil. Furthermore, the activity remained after 1 year's storage in the refrigerator.

The extracted oil was fed at two levels, as shown in Table IV. Since complete protection was obtained at 10 per cent and only partial at 5 per cent, it was apparent that the active substance had not been completely removed by alcoholic extraction. Further

TABLE IV  
*Protective Value of Alcoholic Extract of Soy Bean Oil*

	Supplement	Lard	No. of chicks	No. with disease	Per cent incidence
	<i>per cent</i>	<i>per cent</i>			
Alcoholic extract, 95% alcohol	0.25	21.0	15	1	7
			10	0	0
			10	1	10
			9	0	0
Total.....			44	2	4.5
Alcoholic extract, 95% alcohol after 1 year's storage in refrigerator	0.25	21.0	10	0	0
Oil after extraction with 95% alcohol	10.0	11.0	10	0	0
	5.0	16.0	10	5	50
Oil after further extraction with 95% alcohol, continuous for 2 wks.	5.0	16.0	10	3	33
Alcoholic extract, 75% alcohol	0.15	21.0	10	6	60

extraction of the oil with alcohol in a continuous extraction apparatus for 2 weeks failed to remove any more of the active material, as is demonstrated in Table IV. The residue still showed partial activity.

Extraction of 1.5 kilos of soy bean oil with 75 per cent ethyl alcohol yielded 6.5 gm. of an oily material. When this was added to Diet 108 in the proportion of 1.5 gm. per kilo, it failed to protect, as is shown in Table IV.

*Prevention of the Disease by Non-Saponifiable Matter of Soy Bean Oil*—Since, in preliminary experiments, neither the non-saponifi-

able nor the fatty acid fractions protected chicks, it seemed that the protective factor might have been destroyed by saponification in the presence of air. That this was the case was shown as follows: Three portions of alcoholic extract in alcohol solution were respectively (1) subjected to aeration at room temperature for 48 hours; (2) saponified at room temperature for 18 hours, during which time it was aerated, and then neutralized; (3) saponified as in (2), but in an atmosphere of  $N_2$ , and neutralized before exposure to the air. These products were fed without any further extraction and the results are given in Table V.

TABLE V

*Stability of Protective Factor to Aeration and Saponification*

21 per cent lard in all diets.

Alcoholic extract		No. of chicks	No. with disease	Per cent incidence
	<i>per cent</i>			
Aerated 48 hrs. at room temperature	0.40	10	0	0
Aerated during saponification 18 hrs. at room temperature, neutralized	0.40	10	6	60
Saponified in $N_2$ 18 hrs. at room temperature, neutralized	0.40	9	0	0
	0.75	10	0	0
Saponified in $N_2$ 30 min. at boiling temperature, neutralized	0.40	12	0	0

Both the aerated alcoholic extract and the extract which had been saponified in  $N_2$  protected all of the chicks. Aeration during saponification completely inactivated the extract.

The same results were obtained even when saponification was performed in hot solution, so long as air was excluded. In subsequent experiments, therefore, saponification was always carried out in an oxygen-free atmosphere.

Such precautions, however, did not yield products of constant protective value, as is shown in Table VI.

The conflicting results suggested the possibility of inactivation of the protective factor by impurities in the ether. Freshly distilled low boiling petroleum ether was tried, but, as shown in Table VI, the results with this solvent were no better. However,

when the petroleum ether was purified by treatment with concentrated sulfuric acid for several weeks, followed by washing and distillation, active fractions were consistently obtained.

TABLE VI

*Preventive Effect of Non-Saponifiable Part of Soy Bean Oil*  
21 per cent lard in all diets.

	Supplement	No. of chicks	No. with disease	Per cent incidence
	<i>per cent</i>			
Saponification in presence of air				
Extraction with purified ethyl ether				
Non-saponifiable fraction.....	0.014	10	2	20
Fatty acids.....	0.132	10	8	80
Non-saponifiable fraction.....	0.029	10	7	70
Fatty acids.....	0.113	10	8	80
Saponification in N <sub>2</sub>				
Extraction with purified ethyl ether				
Non-saponifiable fraction.....	0.051	16	0	0
Fatty acids.....	0.165	16	7	44
Non-saponifiable fraction.....	0.042	12	6	50
Fatty acids.....	0.150	11	7	64
Extraction with ordinary petroleum ether				
Non-saponifiable fraction.....	0.042	12	9	75
“ “.....	0.040	13	5	38
Saponification in N <sub>2</sub>				
Extraction with purified petroleum ether				
Non-saponifiable fraction.....	0.235	10	1	10
Remainder, neutralized.....	0.520	10	6	60
Non-saponifiable fraction.....	0.195	10	0	0
Fatty acid “.....	0.189	11	7	64
Water-soluble “.....	0.375	10	8	80
Non-saponifiable “.....	0.024	10	0	0
“ “*.....	0.080	10	0	0
“ “†.....	0.074	10	0	0

\* After extraction with 90 per cent methyl alcohol.

† After extraction with 80 per cent methyl alcohol.

Saponification was carried out by adding 3 gm. of Na dissolved in 100 cc. of 95 per cent alcohol to 15 to 30 gm. of alcoholic extract in 200 cc. of the same solvent, in an atmosphere of N<sub>2</sub>. The reac-

tion was allowed to continue overnight at room temperature. Purified petroleum ether was used to extract the non-saponifiable and fatty acid fractions, which were made up to 50 cc. 10 cc. portions were added to 210 gm. of melted lard and made up into 1 kilo of diet.

As is shown in Table VI, the non-saponifiable fraction protected chicks, but neither the fatty acid nor water-soluble portions had any activity.

#### DISCUSSION

The foregoing experiments demonstrate that this nutritional brain disease is preventable by the addition to the synthetic diet of certain vegetable oils. Furthermore, the protective factor may be extracted, though not completely, by 95 per cent ethyl alcohol. This extract has been stored for a year in the refrigerator without losing its efficacy.

- It has also been shown that the antiencephalomalacic factor is present in the non-saponifiable fraction of soy bean oil. Care must be taken to exclude oxygen during saponification, and to select the proper solvent for extracting the non-saponifiable fraction.

At this stage of fractionation, a 200-fold concentration of the active substance has been obtained. 5 kilos of diet will carry ten to twelve young chicks through a period of from 6 to 7 weeks. If the average daily food consumption per chick is taken as 10 gm., and the protective dose as 0.24 gm. of non-saponifiable fraction per kilo of diet (Table VI), it is seen that approximately 2.4 mg. daily per chick afford complete protection. The soy bean oil preventive diet contained 50 gm. of oil per kilo of diet (Table IV) and furnished roughly 500 mg. daily per chick.

It should be pointed out that Diet 108 containing vegetable oil is not an optimal diet for the chick. Such diets prevent the brain lesions, but do not improve the rate of growth (4) or eliminate other pathological disorders of the young chick, such as perosis.

Since Diet 108 was purposely designed to be low in vitamin E (1) and since the active fractions obtained thus far from vegetable oil would contain this vitamin, the question may be raised as to whether the brain disease may not be a manifestation of vitamin E deficiency in the chick. The evidence is against this. The vitamin E content of Diet 20 has been completely destroyed by treat-



ment with ethereal  $\text{FeCl}_3$  (Waddell and Steenbock (9)), as proved by the usual rat embryo resorption test. This treated diet failed to induce encephalomalacia in chicks. Moreover, as shown in Table I, addition of natural foodstuffs rich in vitamin E did not protect the chicks. It seems certain then that this particular nutritional disease is not a form of vitamin E deficiency.

Dam (10) and Dam and Schönheyder (11) have described a scurvy-like disease of chicks, characterized by hemorrhages into the gizzard and elsewhere, retarded clotting time, and anemia. Protection against this disease was obtained by the non-sterol fraction of the non-saponifiable matter in hog liver fat. It was also afforded by hemp seed oil and certain vegetables.

Whether this factor will prove to be chemically related to the antiencephalomalacic factor cannot be foretold, but there seems to be no relation between the two diseases in respect to symptoms or pathology. Although we have occasionally noted gizzard erosions, hemorrhages, and edema in certain groups of chicks, these were not correlated with encephalomalacic lesions.

It has been stated by Keenan, Kline, Elvehjem, Hart, and Halpin (12) that the factor preventing paralysis encountered on their synthetic Ration 441 is identical with vitamin  $\text{B}_4$ . They also identify this paralysis with the brain degeneration described by Pappenheimer and Goettsch (1); "brain lesions" were observed by them in some of the paralytic chicks that were studied.

In a paper read before the American Chemical Society's Symposium on Vitamins in New York in April, 1935, Elvehjem again attributed encephalomalacia to vitamin  $\text{B}_4$  deficiency. However, he also found that Crisco was effective in prevention, and suggested that, under certain conditions, the water-soluble factor may follow the fat fraction.

Before one draws conclusions as to the identity of a protective factor with vitamin  $\text{B}_4$ , it should be clearly shown that the paralytic disease described by Keenan *et al.* (12) is the same as nutritional encephalomalacia. Since these workers have given no clear description of the pathology, this identity cannot be taken for granted on the basis of symptoms. It is not possible from the symptoms alone to differentiate between the various forms of "paralysis" in growing chicks.

The fact that the preventive factor against nutritional enceph-

alomalacia is present in the non-saponifiable fraction of soy bean oil excludes the possibility that it is vitamin B<sub>4</sub> or any other water-soluble vitamin.

#### SUMMARY

1. A protective factor against nutritional encephalomalacia of chicks is present in certain edible oils such as corn oil, cottonseed oil, hydrogenated cottonseed oil (Crisco), peanut oil, and soy bean oil.

2. The factor, as present in these oils, is thermostable, and resistant to aeration and to ultra-violet irradiation.

3. It can be partially extracted by 95 per cent ethyl alcohol, and this extract is stable.

4. It is present in the non-saponifiable fraction, if due precaution is taken against oxidation during saponification and proper solvents are used for extraction. The fatty acid and water-soluble fractions are inert.

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## ON GLYCOPROTEINS

### II. THE POLYSACCHARIDES OF VITREOUS HUMOR AND OF UMBILICAL CORD\*

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In a previous paper (1) the preparation of a sulfur-free nitrogenous polysaccharide acid from vitreous humor has been described. In the present report a further study of the acid is given. In addition, from umbilical cord two polysaccharide acids have been prepared, one by aqueous extraction, the other by alkaline hydrolysis. The first was sulfur-free and very similar in its properties to the acid from vitreous humor; the second contained sulfate and the amino sugar chondrosamine.

#### EXPERIMENTAL

##### *Analytical Procedures*

Nitrogen was estimated by the micro-Kjeldahl method with (usually) 10 mg. of substance.<sup>1</sup> Sugar analyses were made by the Hagedorn-Jensen method (2) without the  $\text{Zn}(\text{OH})_2$  treatment, unless otherwise stated. Hydrolysis of 5 to 10 mg. samples was carried out with 2 cc. of 2 N HCl or  $\text{H}_2\text{SO}_4$  in sealed tubes in a boiling water bath for 8 to 10 hours, which was found to be the

\* Reference (1) is to be considered Paper I of the series. A preliminary report of this paper was presented by title at the meeting of the American Society of Biological Chemists at Washington, March, 1936.

<sup>1</sup> When  $\text{H}_2\text{O}_2$  was used to assist the Kjeldahl oxidation of amino sugars or the polyuronic acids, nitrogen values (agreeing with those obtained by the micro-Dumas procedure) about 4 parts per 100 higher than those reported here were obtained.

optimal time. Longer hydrolysis led to slow disappearance of sugar. Since the uronic acid component was largely destroyed by the hydrolytic treatment, the values for the reducing material content are probably too low. In a control experiment with 0.82 mg. of glucuronic acid and 0.90 mg. of glucosamine (1.08 mg. of glucosamine hydrochloride) and a mixture of the two, the uronic acid gave a reduction equivalent to 34 per cent of the theoretical after  $\text{Zn}(\text{OH})_2$  precipitation, 52 per cent without it; the amino sugar gave 98 and 100 per cent respectively, and the mixture in each case gave a reduction equal to the sum of the two components.

Amino sugar was estimated by our modification<sup>2</sup> of the method of Elson and Morgan (3), 2 to 10 mg. of substance being hydrolyzed as for the sugar estimation. The ratio of amino sugar to total nitrogen was used as an indication of the purity of the preparation.

Acetyl was determined by the method of Kuhn and Roth (4) with a simpler apparatus. Correct values were obtained when *p*-toluenesulfonic acid was used for saponification:<sup>3</sup> acetyl in N-acetylglucosamine found, 19.2 per cent; theoretical, 19.4 per cent.

Moisture content was estimated by heating 20 mg. samples in small crucibles at 90° to constant weight; ash was determined in the same sample by igniting at bright red heat for 2 to 3 hours in an electric crucible furnace. Drying at 90° did not remove all the moisture present. A sample (see Table I) when dried for 5 days at 100° *in vacuo* over  $\text{P}_2\text{O}_5$  lost more than 9.5 per cent of its weight as compared with 2.7 per cent by the usual procedure. The residue was extremely hygroscopic.

Equivalent weight was obtained by titration of 20 mg. samples dissolved in 10 drops (about 0.5 cc.) of water with 0.01 N NaOH with phenolphthalein as the indicator.

Uronic acid was determined in 10 to 15 mg. samples by the

<sup>2</sup> The procedure will be described in detail elsewhere.

<sup>3</sup> On alkaline hydrolysis, our purest preparations of the polysaccharide from vitreous humor yielded 1.8 to 2.0 moles of acid per atom of nitrogen; in a previous paper (1) we therefore gave the acetyl value as 2 moles per equivalent weight of polysaccharide acid (each equivalent containing 1 atom of nitrogen). However, with a pure sample of N-acetylglucosamine, prepared according to Zuckerkandl and Messiner-Klebermass (5), a value of 2 moles of acid was found. With pure glucose under the same conditions 0.8 mole of acid was found. The hydrolysis with methyl alcoholic NaOH therefore could not be used with the sugars.

Lefèvre-Tollens method (6), as applied to small quantities by Burkhart, Baur, and Link (7). The apparatus was somewhat simplified and an efficient reflux condenser was employed to prevent the transfer of acetic acid in the air stream. A blank equivalent to 0.70 cc. of 0.01 N Ba(OH)<sub>2</sub> was found with no sample present; with 15.5 mg. of acetylglucosamine the blank was 1.00 cc. The latter figure was used in calculating the results of the determinations. With crystalline glucuronic acid (5.80 mg.) a recovery of 102 per cent was obtained after deduction of the blank value.

All analytical values given are corrected for ash and moisture (loss at 90°) unless otherwise specified. •

#### *Preparation and Properties*

*Vitreous Humor*—The polysaccharide acid from vitreous humor (hyaluronic acid) was prepared as described previously (1). The nitrogen content did not decrease after repeated precipitation of the substances with glacial acetic acid, but did decrease when the powders, dissolved in a small amount of water and neutralized, were treated with kaolin, Lloyd's reagent, or with zinc acetate followed by neutralization. With the latter procedure, samples were obtained in which the amino sugar as estimated accounted for 95 to 100 per cent of the total nitrogen.<sup>4</sup> A detailed example will illustrate the procedure.

The acetone precipitate of the vitreous humor from 200 cattle eyes (5.9 gm.) was extracted in the cold with one 200 cc. and two 100 cc. portions of 90 per cent acetic acid over 2 to 4 days. After several washings with alcohol the residue was three times extracted with 200 cc. of water, neutralized, and centrifuged. The combined supernatant solutions were acidified with acetic acid and precipitated with 6 volumes of alcohol. The precipitate was washed with alcohol, taken up in 150 cc. of water, poured into 12 volumes of glacial acetic acid, washed with alcohol until free of acetic acid, taken up in 100 cc. of water, neutralized, and centrifuged. The supernatant fluid was acidified with HCl and immediately poured into 12 volumes of glacial acetic acid. After repeatedly washing with alcohol, acetone, ether, and drying *in vacuo*, 1.035 gm. of a

<sup>4</sup> Somewhat less than the theoretical values for amino sugar were often found because of disappearance of amino sugar upon neutralization of the hydrolysate.

white, stringy powder were obtained. The nitrogen content was 3.78 per cent.

A part of this material (487 mg.) was dissolved in 50 cc. of water, neutralized, and divided into two portions. The first, after the addition of 1 cc. of 10 per cent acetic acid, was shaken with about 1 gm. of Lloyd's reagent, centrifuged, the supernatant solution neutralized to phenolphthalein, again shaken with Lloyd's reagent, and centrifuged. The supernatant solution was precipitated with 4 volumes of alcohol, redissolved in 20 cc. of water, brought to 0.2 N HCl, and reprecipitated with 10 volumes of glacial acetic acid. The yield was 189.5 mg.; the nitrogen content was 3.28 per cent.

To the second portion of the polysaccharide solution, diluted with an equal volume of water, 20 cc. of 4 per cent zinc acetate were added; N NaOH was added until a clear supernatant solution was obtained. The solution was precipitated with alcohol and acetic acid as above. The yield was 154.7 mg.; the analysis (Preparation A) is given in Table I. The ash content was 1.38 per cent.

To avoid the use of high concentrations of acetic acid, the removal of protein was attempted by denaturation by shaking with chloroform or mixtures of chloroform and amyl alcohol, as in a method used by Sevag and others (8). In this way a polysaccharide of high viscosity (see p. 697) was obtained. This procedure is illustrated by the following preparation.

The aqueous extract from 3.29 gm. of acetone-dried vitreous humor was precipitated by 5 volumes of alcohol, and the precipitate was dissolved in 100 cc. of water and neutralized. The solution was then twice shaken for about 20 hours with 25 to 30 cc. portions of chloroform. The aqueous solution was separated at the centrifuge and precipitated by alcohol, redissolved, and again treated with chloroform. The clear supernatant liquid was precipitated by acetone after the addition of some salt (sodium acetate). The precipitate was redissolved in 50 cc. of water, neutralized, and precipitated by 3 volumes of alcohol in the presence of a few drops of glacial acetic acid to complete the flocculation. The yield was 786 mg. (Preparation C); the analysis gave nitrogen 4.78 per cent, ash 5.15 per cent. Solutions of this material were very viscous.

The further removal of nitrogenous contaminants was attempted by treatment with  $\text{Zn}(\text{OH})_2$ . A part (700 mg.) of the above product in 200 cc. of water was treated with 25 cc. of 4 per cent zinc acetate and alkali. After repeated acidification, neutralization, and centrifugation, the solution was brought to 0.1 N HCl and precipitated immediately with 5 volumes of alcohol as a homogeneous fibrous mass. The substance (Preparation D) gave the following analysis: nitrogen 3.30 per cent, acetyl 10.9 per cent, total sugar 69.5 per cent, hexuronic acid 46.6 per cent, equivalent weight 443, ash 1.69 per cent;  $[\alpha]_D^{25}$  (neutralized solution) =  $-51^\circ$ . The viscosity (see p. 697) was not greatly lowered by this prolonged treatment. The removal of zinc from this viscous material was extremely difficult; an intermediate sample of this preparation contained 18.61 per cent ash, consisting mainly of zinc salts and oxide.

For the isolation of the amino sugar, 800 mg. of mixed preparations of crude hyaluronic acid were hydrolyzed for 6 hours with 30 cc. of 18 per cent HCl in the presence of  $\text{SnCl}_2$ .<sup>5</sup> After removal of tin and evaporation to a syrup, the material was leached with hot 50 per cent methanol. Acetone was added to the extract until a permanent turbidity was produced, and the material was kept in the cold with occasional scratching of the sides of the vessel. A snow-white crystalline precipitate weighing 292 mg. was obtained. After reprecipitation from hot 75 per cent methanol with acetone in the same manner, 181 mg. were collected in the first fraction.

$\text{C}_6\text{H}_{11}\text{O}_6\text{NCl}$  (hexosamine hydrochloride). Calculated, N 6.53, equivalent weight 214.6; found, N (Dumas<sup>6</sup>) 6.37, 6.40, equivalent weight (formol titration) 218.7;  $[\alpha]_D^{20}$  (at equilibrium) =  $+78.5^\circ$ .

The equilibrium rotation of the second fraction was  $[\alpha]_D^{24} = +73.7^\circ$ . The first fraction further recrystallized gave a value of  $[\alpha]_D^{23} = +75.5^\circ$ . A value of  $+72.5^\circ$  was found for known glucosamine hydrochloride. A phenylosazone was prepared from the recrystallized first fraction which after two recrystallizations from

<sup>5</sup> For the preparation of the amino sugar hydrochloride, Levene's method (9) of hydrolyzing in the presence of  $\text{SnCl}_2$  is in our experience of greater advantage than any other method tried.

<sup>6</sup> This analysis was made by Mr. William Saschek of the Department of Biological Chemistry.



aqueous pyridine melted at  $210^{\circ}$  (corrected). The sugar was obviously glucosamine. The total yield of material before recrystallization was 76 per cent of the theoretical amount present, assuming pure starting materials.

A mixed sample of hyaluronic acid (400 mg. not highly purified) when oxidized with nitric acid according to the procedure given by van der Haar (10) yielded saccharic acid (isolated as the acid potassium salt which was identified by its crystal habit and by formation of the typical thallium salt (10)), but no mucic acid. This indicates the presence of glucuronic acid and the absence of galactose and galacturonic acid.

*Umbilical Cord*—From umbilical cord Levene and López-Suárez (11) obtained by alkaline hydrolysis of the whole cord a complex sugar, the barium salt of which contained 3.16 per cent sulfur. In the present work two different complex carbohydrates were obtained, one by aqueous extraction, a sulfate-free material apparently present in Wharton's jelly, the other by alkaline extraction of the residue, a sulfuric acid ester, probably from the connective tissue elements. The first compound was similar to the polyuronic acid from vitreous humor and contained glucosamine; the second was characterized through its amino sugar as chondroitin sulfuric acid.

For the preparation of the polyuronic acid, eight to ten fresh human cords were washed free of adhering blood and kept under acetone until used. They were then ground in a meat grinder or a small Wiley mill. The powder was extracted with 90 per cent acetic acid until no hematin appeared in the extract. The residue was washed with alcohol and extracted with 200 to 100 cc. portions of water for several days in the cold; the extract was neutralized from time to time. The combined aqueous extracts were precipitated by alcohol and glacial acetic acid as described for the vitreous humor material. The yield was about 1.3 gm. from 62 gm. of dried cords. Tests for sulfate and the biuret reaction were negative. The Molisch reaction and Tollens' naphthoresorcinol reaction (for uronic acid) were positive. The nitrogen content was 4.20 per cent; equivalent weight, 490. After treatment with  $\text{Zn}(\text{OH})_2$  and subsequent reprecipitation successively by alcohol and glacial acetic acid, the product contained nitrogen 3.21 per cent, acetyl 11.5 per cent, total sugar 62.2 per cent, hexosamine

40.3 per cent, equivalent weight 441, ash 0.11 per cent. Kaolin and Lloyd's reagent were not as effective in lowering the nitrogen.

All samples of this substance, prepared as described above, were found to be contaminated with glycogen (iodine color reaction). To remove glycogen, 777 mg. of material purified by treatment with  $\text{Zn}(\text{OH})_2$  as above were incubated in neutral 1 per cent solution with 1 cc. of filtered saliva in the presence of toluene at  $37^\circ$  until the iodine color reaction was negative. The solution was precipitated by alcohol, reprecipitated by glacial acetic acid, and finally precipitated from 50 cc. of 0.1 N HCl by alcohol. The product (675 mg.) gave the analysis shown in Table I, Preparation B. The ash content was 0.36 per cent;  $[\alpha]_D^{25} = -65^\circ$  in neutral solution.

The amino sugar of this acid was isolated by the procedure used for hyaluronic acid (see above). From 800 mg. of impure starting material a total of 221 mg. was obtained as the hydrochloride. After reprecipitation, 171 mg. of snow-white crystalline substance were collected in the first fraction. The equivalent weight (formol titration) was 217.8 (theory for hexosamine hydrochloride, 214.6).  $[\alpha]_D^{22}$  at equilibrium was  $+70.3^\circ$ . The sugar was obviously glucosamine.

A mixed sample (500 mg.) of the polyuronic acid from umbilical cord, oxidized with nitric acid (10), yielded acid potassium saccharate (155 mg.), but no mucic acid. This indicates the presence of glucuronic acid and the absence of galactose and galacturonic acid. Methoxyl was not present.<sup>6</sup>

The residue from the aqueous extraction of the cords still gave a strong Molisch reaction. It was three times further extracted with water, the second extract giving a negative Molisch reaction. The residue was then allowed to stand with 7.2 per cent NaOH at room temperature for 2 days; the extract was evaporated on the steam bath with an excess of  $\text{BaCO}_3$ , leached with water, and the supernatant liquid precipitated with 2 volumes of alcohol. The precipitate was washed with alcohol of increasing concentration, dissolved in a small amount of water, neutralized with  $\text{Ba}(\text{OH})_2$ , and precipitated with 10 volumes of glacial acetic acid. After freeing the slimy precipitate from acetic acid with alcohol, acetone, and ether, a dry white powder weighing 1.5 gm. was obtained from 76 gm. of dried cords. The analysis gave nitrogen 2.86 per

cent, acetyl 4.04 per cent, total sugar 62.2 per cent, hexosamine 11.6 per cent, equivalent weight 836, acid-hydrolyzable sulfate (as sulfur) 2.53 per cent, ash 6.36 per cent. The material gave a strong iodine color test for glycogen; after incubation with saliva, the corrected nitrogen content was 4.00 per cent.

About 1.4 gm. of this material were hydrolyzed according to Freudenberg and Eichel (12). After treatment with norit "A" and evaporation, the residue was taken up in hot 50 per cent methanol and precipitated with acetone. The oily material was repeatedly reprecipitated from hot 75 per cent methanol by acetone until a uniform white crystalline substance (68.1 mg.) was obtained.<sup>5</sup> The nitrogen content (Dumas<sup>6</sup>) was 6.38 per cent; theory for hexosamine hydrochloride, 6.53 per cent. The substance was difficult to burn, and was mixed with  $\text{KClO}_3$  for the combustion. At equilibrium  $[\alpha]_D^{24}$  was  $+94.0^\circ$ . The substance was therefore chondrosamine hydrochloride ( $[\alpha]_D^{25} = +95^\circ$  (9)), predominantly in the  $\beta$  form.  $[\alpha]_D^{23} = +74.7^\circ$  16 minutes after solution (cf. Levene (9) p. 20).

#### *Physical Properties of the Two Polyuronic Acids*

The two acids were obtained as snow-white fluffy powders and formed water-clear solutions. They were precipitated from concentrated aqueous solutions with alcohol or glacial acetic acid as a stringy meshwork resembling asbestos or cotton fibers. Their solutions did not become turbid on acidification with acetic acid. In aqueous solutions they did not pass through a collodion membrane of high permeability. When a concentrated aqueous solution was pressed from a fine cannula into glacial acetic acid, long fibers could be spun; these fibers were doubly refractive in polarized light.

The aqueous solutions were viscous materials from umbilical cord being more viscous than comparable products from vitreous humor. The viscosity of the solutions was dependent upon the treatment of the material during preparation. The solution of an alcohol precipitate (with which glacial acetic acid had never been used as a precipitant) had a high viscosity; that of a glacial acetic acid preparation had greatly lost this property, and subsequent reprecipitation by alcohol did not restore it.

In one experiment the viscosities of two preparations of the

polyuronic acid from umbilical cord were compared; the preparations were identical except that one had been precipitated by glacial acetic acid (ash 1.17 per cent, nitrogen 4.20 per cent), and the other by alcohol (ash 6.13 per cent, nitrogen 5.18 per cent). The solutions (in Ringer's solution) were neutralized and made up to 0.25 per cent. In the Ostwald viscosimeter at 20.0–20.5°, the solution of the alcohol precipitate had a viscosity, relative to water, of 12.9 to 13.2, while the acetic acid precipitate gave figures of 2.16 to 2.19.

Similarly hyaluronic acid, prepared in the usual way with glacial acetic acid, had in 0.25 per cent neutral solution a relative viscosity of 1.205, while samples prepared by the chloroform method and precipitated by alcohol (Preparations D and C, see above) had relative viscosities of 3.12 and 3.575 under the same conditions. These three hyaluronic acid preparations had, respectively, the following nitrogen contents: 3.86, 3.30, and 4.78 per cent; the ash contents were 0.21, 1.69, and 5.15 per cent respectively.

The supernatant glacial acetic acid on evaporation and drying with alcohol and ether gave a small amount of material devoid of any measurable viscosity.

#### *Artificial "Mucoids"*

The classical method of preparing mucoids has been the precipitation from aqueous solution by addition of acetic acid to a concentration of 2 per cent. Thus Mörner (13) and others after him obtained from vitreous humor a "mucoid" having 12.25 per cent nitrogen and 1.19 per cent sulfur. By the precipitation of diluted filtered vitreous humor, according to Mörner's procedure, we have obtained similar products.

From mixtures of either of our polyuronic acids with various proteins similar preparations were obtained which did not materially change their composition on reprecipitation. Such "mucoids" were made from both the acids with crystallized egg albumin, with the globulin edestin, and with the basic protein globin from horse hemoglobin. A typical procedure is given. A solution (5 cc. containing 59 mg. of protein per cc.) of crystallized egg albumin was mixed with 5 cc. of 0.25 per cent neutral solution of umbilical cord polysaccharide; acetic acid was added to a concentration of

2 per cent. The precipitate was centrifuged off, redissolved in 10 cc. of water containing a few drops of  $N$  NaOH, and reprecipitated from 2 per cent acetic acid. After washing and drying, 84 mg. of material were obtained, which gave: nitrogen 12.80 per cent, total sugar (after  $Zn(OH)_2$  precipitation) 14.5 per cent, hexosamine 6.4 per cent,<sup>4</sup> ash 0.47 per cent. With edestin and the same umbilical cord preparation a similar material was obtained: nitrogen 13.23 per cent, total sugar (after  $Zn(OH)_2$  precipitation) 18.9 per cent, hexosamine 9.7 per cent,<sup>4</sup> ash 0.00 per cent; with edestin and hyaluronic acid: nitrogen 13.4 per cent, total sugar (after  $Zn(OH)_2$  precipitation) 17.5 per cent, hexosamine 8.6 per cent,<sup>4</sup> ash 0.00 per cent. For the latter two preparations, the edestin was dissolved in 2 per cent acetic acid. Neither the polyuronic acids nor the proteins employed were precipitated from 2 per cent acetic acid when not mixed.

#### DISCUSSION

The purest of the polyuronic acid preparations described were substantially free from nitrogenous contaminants. The materials from both sources appeared homogeneous, and had the same composition when obtained by different procedures.<sup>7</sup> They contained nitrogen, hexosamine, and hexuronic acid in the ratio of approximately 1 equivalent per equivalent weight. In most of them acetyl appeared in the ratio of about 1.2 equivalents per equivalent weight. From the analytical figures (Table I) it is evident that the samples when dried in the routine manner contain about 3 moles of water per equivalent weight. One more highly desiccated sample, weighed as quickly as possible, seemed to contain about 2 moles of water. Table II expresses the same analytical figures as equivalents per equivalent weight. It does

<sup>7</sup> This is in contrast to our unpublished data on the polysaccharides of gastric mucin, preparations of which have hexuronic acid contents varying from 0 to over 25 per cent, depending upon the manner of preparation; all seem to have a large amount of galactose, as judged by the ability to form mucic acid. Gastric mucin apparently contains a mixture of polysaccharides which is difficult to separate. One fraction, which Dr. K. Landsteiner of the Rockefeller Institute kindly tested, was highly active for the blood Group A substance (positive precipitin reaction at a dilution of 1:4,000,000).

not seem likely that there is any other constituent present in the molecule.

TABLE I  
*Comparison of Analyses of the Two Polyuronic Acids*

	Nitrogen	Hexos- amine	Acetyl	Hex- uronic acid	Equiva- lent weight
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Found, vitreous humor					
Preparation A, ash-free . . . . .	3.11	39.3	11.7	45.3	424
Found, umbilical cord					
Preparation B, ash-free, by usual procedure . . . . .	3.11*	40.1	12.4	45.1	421
Found, umbilical cord					
Preparation B, ash-free, corrected for maximum moisture content obtained . . . . .	3.35†	43.2	13.4	48.6	391
Calculated for anhydride of acetyl- hexosamine and hexuronic acid + 2H <sub>2</sub> O . . . . .	3.37	43.1	10.4	46.8	415
Calculated for above anhydride + 3H <sub>2</sub> O . . . . .	3.24	41.4	9.9	44.8	433

\* When H<sub>2</sub>O<sub>2</sub> was used to assist the Kjeldahl oxidation, a value of 3.23 was obtained.

† When H<sub>2</sub>O<sub>2</sub> was used to assist the Kjeldahl oxidation, a value of 3.48 was obtained.

TABLE II  
*Figures of Table I Expressed As Equivalents per Equivalent Weight*

	Nitrogen	Hexos- amine	Acetyl	Hexuronic acid
Vitreous humor				
Preparation A . . . . .	0.94	0.93	1.15	0.99
Umbilical cord				
Preparation B . . . . .	0.94*	0.94	1.22	0.98

\* When H<sub>2</sub>O<sub>2</sub> was used to assist the Kjeldahl oxidation, a value of 0.98 was obtained.

The hyaluronic acid preparations were not contaminated with glycogen (by the iodine color test) as were comparable preparations from umbilical cord. With the latter after incubation with

saliva, the analytical figures were practically the same as with hyaluronic acid preparations.

Combustion of vitreous humor Preparation A after drying *in vacuo* for 3 days at 70° gave C 44.1 per cent, H 5.51 per cent.<sup>6</sup> Umbilical cord Preparation B gave C 44.2 per cent, H 5.61 per cent.<sup>6</sup> Theory for  $C_{14}H_{21}O_{11}N$  (acetylhexosamine hexuronic acid anhydride): C 44.3 per cent, H 5.58 per cent. Samples dried in this manner and weighed quickly in closed containers were therefore practically water-free.

The possible presence of pentose, based on positive Bial and Schiff reactions, in hyaluronic acid has already been reported (1). Schiff's aniline reaction (10) is said to be specific for pentoses. The latter reaction was positive with both the vitreous humor and the umbilical cord preparations, but under the same conditions we also obtained positive reactions on pure glucuronic and galacturonic acids. Therefore, we now doubt the presence of pentose.

From vitreous humor and from umbilical cord Levene and coworkers (9) obtained substances which they designated as mucoitin sulfuric acids.<sup>8</sup> These contained (as barium salts) respectively, 3.63 and 4.07 per cent sulfur. The other typical constituent of vitreous humor, the supporting structure, contains some reducing sugar (11 per cent) including about 3 per cent amino sugar, but does not contain any appreciable amounts of sulfate. Its concentration in the vitreous humor (16 mg. per 100 cc. by our results, or 15 to 19 mg. per 100 cc. according to Friedenwald and Stiehler (14)) is too low to explain the high sulfate content found by Levene.

Solutions of hyaluronic acid, other complex sugar acids, and mucoids are effective protective colloids and tenaciously hold insoluble inorganic materials in colloidal solution (*cf.* the removal of  $Zn(OH)_2$  above). In cases where barium or lead had been used in the preparation we found it very difficult to remove the barium sulfate or lead sulfide from solution. Barium sulfate so held in suspension would of course precipitate after acid hydrolysis and appear as "hydrolyzable" sulfate.

<sup>8</sup> The only source from which we isolated a substance having the composition of a mucoitin sulfuric acid, confirming Levene's finding, was the cornea. The hexosamine was isolated and characterized as glucosamine. A detailed report is in preparation.

In the case of the umbilical cord material, it seems probable that Levene was dealing with a mixture of the polyuronic acid and the chondroitin sulfuric acid. It is conceivable that after acid hydrolysis he isolated glucosamine hydrochloride from the polyuronic acid, since chondrosamine hydrochloride is more readily soluble than glucosamine hydrochloride. We found that a mixture of equal parts of the hydrochlorides of glucosamine and chondrosamine<sup>9</sup> after two recrystallizations had a specific rotation at equilibrium of  $[\alpha]_D^{25} = +77.2^\circ$ , corresponding to a mixture of about 21 per cent chondrosamine and 79 per cent glucosamine, as hydrochlorides.

The viscosity of the aqueous solutions of these polyuronic acids is probably of biological significance. The viscosity of hyaluronic acid is apparently responsible for the turgor of the vitreous humor. Solutions of material from umbilical cord were always much more viscous than comparable preparations from vitreous humor. The viscosity of materials from both sources was markedly lowered after precipitation by glacial acetic acid, although the analytical figures of the products were not necessarily different. Purification of the material by more gentle means produced only a slight lowering of the viscosity, far less than that caused by treatment with glacial acetic acid.

According to Staudinger (16) viscosities as high as those reported here can be possible only if the molecules are arranged in long thread-like aggregates; such a concept in regard to these acids is in accord with their form and behavior upon precipitation. The loss of viscosity after precipitation with glacial acetic acid is apparently not due to a change in the chemical make-up of the molecules, but to a partial breakdown of these long aggregates.

The materials obtained from mixtures of these polyuronic acids with various proteins upon precipitation from 2 per cent acetic acid have been called artificial mucoids, since they appear very similar in composition and properties to the "mucoids" obtained by this (Mörner's) method from various natural sources (13). Their formation under these conditions shows that the precipitation of the "mucoids" from natural solutions containing protein

<sup>9</sup> This was prepared by the procedure of Levene and La Forge (15) from chondroitin sulfuric acid furnished us through the courtesy of Dr. David Klein of the Wilson Laboratories, Chicago.



and a polyuronic acid is no proof for their existence as such in the native solution. They are probably salts, and in neutral or alkaline solutions are dissociated;<sup>10</sup> the sugar components of the "mucoids" of at least two tissues, vitreous humor and the outer portion of umbilical cord, can be separated without hydrolytic procedures.

#### SUMMARY

Further purification of hyaluronic acid has been achieved by the use of  $\text{Zn}(\text{OH})_2$ . A very similar material has been obtained by aqueous extraction of acetone-dried umbilical cords. The amino sugar of these polyuronic acids has been isolated and characterized as glucosamine; the uronic acid is apparently glucuronic acid. These two constituents appear in the ratio of 1 mole per equivalent weight. Acetyl is present in slightly higher concentration. Preparations were obtained free from nitrogenous impurities.

A sulfate-containing sugar acid was prepared by alkaline hydrolysis of the water-extracted umbilical cord residue; its amino sugar was isolated and identified as chondrosamine.

The discrepancy between these data and those of Levene and coworkers (9) is discussed.

The viscosity of solutions of the sulfate-free polyuronic acids was found to be dependent upon the treatment during preparation. Solutions of preparations from umbilical cord have much higher viscosities than comparable materials from vitreous humor. The high viscosity of aqueous solutions and the behavior upon precipitation indicate that the molecules of these polyuronic acids are arranged in long thread-like aggregates.

Artificial "mucoids" have been prepared from mixtures of the polyuronic acids with various proteins.

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<sup>10</sup> It was noted that the complexes with an albumin dissociate more readily than those with a globulin; the latter require a more alkaline medium for solution. Similar complexes of great stability were obtained with chondroitin sulfuric acid. Such a salt with gelatin formed a doubly refractive elastic mat. With edestin a material resembling fibers of elastin was obtained.

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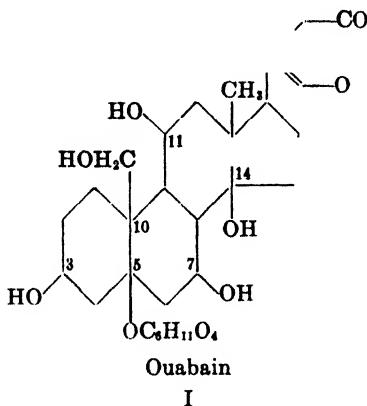
# OUABAIN

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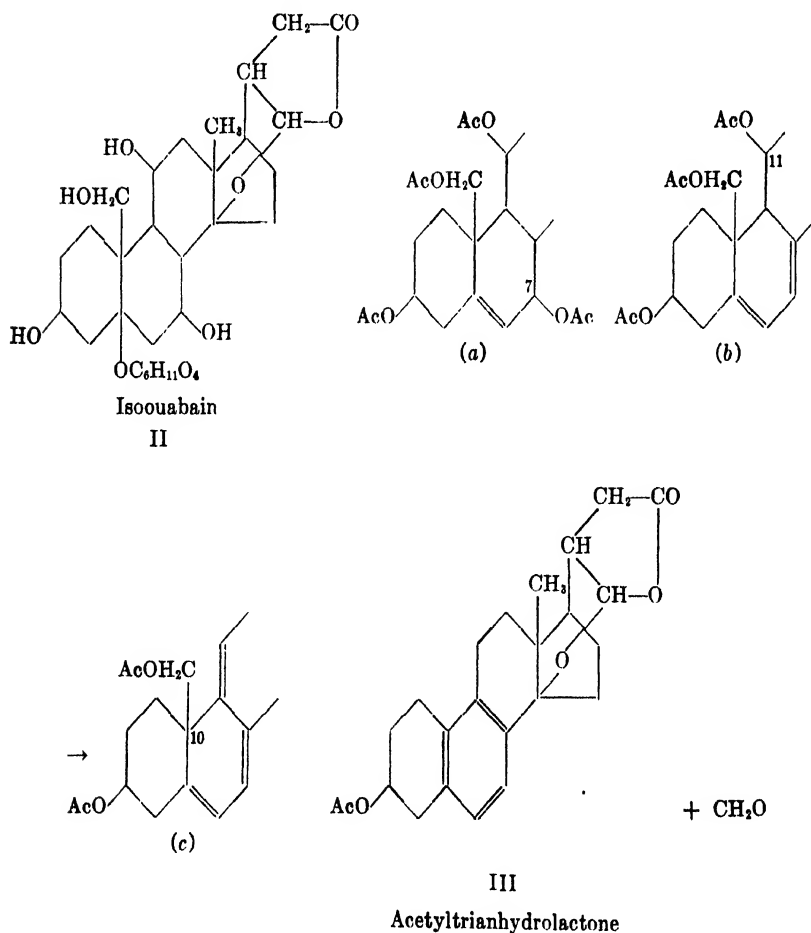
According to views set forth elsewhere by one of us (1), the results obtained by Arnaud (2) and by Jacobs and Bigelow (3, 4) in their investigations of ouabain (*g*-strophanthin) are most satisfactorily interpreted in terms of Formula I for the glycoside. This is in accord with evidence, presented for the most part by



Jacobs and Bigelow, that ouabain yields a dihydro derivative, gives a positive Legal reaction, forms an iso compound, and gives with acetic anhydride and zinc chloride a heptaacetylanhydroglycoside, a tertiary hydroxyl group at C<sub>14</sub> evidently being eliminated in the course of the latter reaction. It is supposed that three of the acetyl groups enter the sugar residue and that three of the nuclear hydroxyl groups which become acetylated are secondary, while the remaining one is a primary group, the angular methyl group at C<sub>10</sub> being hydroxylated.

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The reason for suggesting that the hydroxyl groups occupy the specific positions indicated is that this particular arrangement provides a reasonable explanation of the cleavage of formaldehyde from the genin moiety in the course of the acetolysis of heptaacetyldesoxydihydroouabain (3) and of isoouabain (4). As shown in the accompanying formulas, the latter reaction is thought to proceed through a succession of dehydrations or cleavages, probably initiated by the elimination of the tertiary rhamnoside group at C<sub>5</sub>. It is assumed that the new double bond of (a)



activates the secondary, acetylated group at C<sub>7</sub> and promotes its elimination because of the tendency to form the conjugated system present in (b). That an  $\alpha,\beta$ -unsaturated secondary carbinol group is about as subject to dehydration as an ordinary tertiary group is clearly indicated by the ready dehydration of epiallocholesterol (5), and by the conversion of gitoxigenin into a dianhydro compound with cold concentrated hydrochloric acid (6). Following the formation of (b) the conjugated system can be extended still further by the loss of acetic acid, giving (c).<sup>1</sup> In this phase the primary carbinol group at C<sub>10</sub> would be susceptible to a Wagner-Meerwein rearrangement and the bond by which it is linked to the aglycone nucleus would be weakened by the presence of the two double bonds in the  $\alpha,\beta$  position. The expulsion of this group as formaldehyde with aromatization of Ring B may be due to a combination of these special features of structure, the weakened bond being ruptured before a true rearrangement can proceed to completion. An analogous elimination of a similar carbon residue occurs in the acetolysis of  $\alpha$ -methylmorphimethine to methylmorphol and ethanoldimethylamine (8).

The end-product of the acetolysis was characterized by Jacobs and Bigelow as an acetyltrianhydrolactone, and most of the properties reported for the compound are adequately interpreted by Formula III. The lactone ring of the desacetyl compound can be opened by hydrolysis and the acid group esterified. The ester on oxidation gives a ketolactone, both a nuclear secondary alcoholic group and that in the side chain being attacked. This indicates that the entire isoaglycone grouping is still intact and that one of the original secondary hydroxyl groups, probably at C<sub>3</sub>, survives the anhydro reaction. The properties of the acetoxylactone (C<sub>24</sub>H<sub>30</sub>O<sub>4</sub>) obtained (3) from heptaacetyldeoxydihydroouabain can be accounted for by means of a similar formula, but there is in

<sup>1</sup> By similar reasoning it would be expected that a substance having the formula suggested by Tschesche and Haupt (7) for convallatoxigenin would easily form a dianhydro compound, for in the monoanhydro derivative the tertiary hydroxyl group at C<sub>8</sub> would be activated by the double bonds at C<sub>7</sub>-C<sub>11</sub> and C<sub>14</sub>-C<sub>15</sub>, and the conjugated system could be extended further by the elimination of water at C<sub>7</sub>-C<sub>8</sub>. An alternate suggestion is that the hydroxyl group be transposed from C<sub>8</sub> to the angular methyl group at C<sub>10</sub>, as pictured for ouabain. The hindrance at this position probably would be sufficient to prevent the ready formation of a dibenzoyl derivative.

each case one observation with which the present interpretation may at first appear out of harmony. Although an aromatic nucleus usually is not susceptible to hydrogenation in the presence of platinum oxide catalyst, Jacobs and Bigelow found that each degradation product absorbed 3 moles of hydrogen. They state, however, that the product from isouabain was wholly unattacked in neutral solvents but that it was slowly hydrogenated in glacial acetic acid solution. The behavior was said to be reminiscent of that of trianhydrostrophanthidin, a compound generally regarded (9, 10) as having a benzenoid ring. There is also a close parallel to the case of estrone, for with this (albeit phenolic) substance the aromatic nucleus resists hydrogenation in neutral solvents but is attacked, usually even before the carbonyl group, when the reaction is conducted in acetic acid solution or in alcohol containing hydrochloric acid (11).

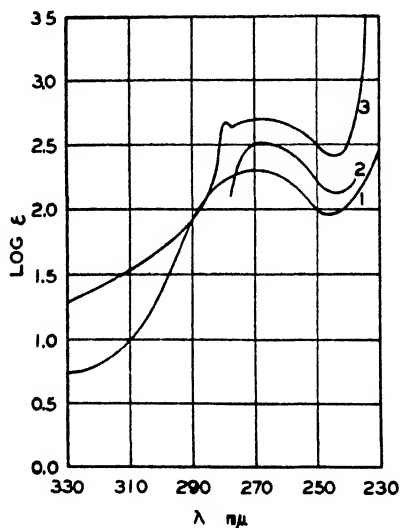


FIG. 1. Curve 1, acetyltrianhydrolactone from isouabain in alcohol; Curve 2, neoergosterol in ether (Inhoffen (12)); Curve 3, dihydrotrianhydrostrophanthidin in alcohol (Elderfield and Rothen (9)).

All things considered, the observations of Jacobs and Bigelow regarding the hydrogenations do not exclude the suggested struc-

tures, but they clearly make it necessary to seek other evidence on the important question of whether or not the three double bonds are contained in the same nucleus. In comparable cases the most reliable means of deciding this subtle point is from the absorption spectrum, and this method has been applied to the case at hand. A sample of the acetyltrianhydrolactone from isouabain was prepared and purified according to Jacobs and Bigelow (3); m.p. 243–245°, uncorrected,  $[\alpha]_D = -27.6^\circ$  in pyridine. The absorption curve shown in Fig. 1 was kindly determined by Mr. W. H. Avery. Included in the graph are the curves for dihydrotrianhydrostrophanthidin (9), and neoergosterol (12), and the similarity in the shape of the curves and the positions of the maxima is at once apparent. With both of the latter compounds Ring B very probably is aromatic, the evidence being particularly decisive in the case of neoergosterol (13). Consequently, a formula such as Formula III is strongly indicated for the isouabain degradation product. The observed absorption maximum at 270  $m\mu$  is, according to available information, characteristic of a benzenoid ring. Perhaps the most pertinent example of a compound having three conjugated double bonds distributed in more than one nucleus is dehydroergosterol, which shows an absorption maximum at 320  $m\mu$  (14).

An attempt was made to obtain further evidence by titrating the acetyltrianhydrolactone with perbenzoic acid, but the results were not entirely satisfactory. A solution of 74.1 mg. of the material in 10 cc. of chloroform was mixed with 10 cc. of a chloroform solution containing an excess of perbenzoic acid, and after standing at 5° it was compared from time to time with a similarly diluted blank solution by titration of a 5 cc. portion with 0.0980 N sodium thiosulfate. The results were as follows:

Time, hrs. . . . .	72	136	237
Blank, cc. . . . .	23.45	23.25	22.30
Titer, cc. . . . .	22.48	21.65	20.58
Atoms of oxygen . . . . .	1.0	1.7	1.8

Although some reaction clearly occurs, it is considerably slower and less extensive than would be expected if the compound contains three non-benzenoid double bonds. Ergosterol, for example,



consumes 3.08 atoms of oxygen in 40 hours at 0° (14). In the present case there is a deficiency of over 1 atom of oxygen even after a period six times as long, and it seems possible that the observed effect is due to some reaction other than addition to the double bonds, such as a dehydrogenation (15), or some disturbance of the isoaglycone grouping. A knowledge of the behavior of other cardiac aglycone derivatives would be illuminating in this connection.

At present it may be said that the absorption spectrum definitely indicates that the three double bonds are present in a conjugated system and that they probably constitute a benzenoid ring. Their partial or sluggish response to hydrogenation and to the action of perbenzoic acid is very difficult to reconcile with the idea that they are conjugated but not aromatic, and the data as a whole lend definite support to the structures suggested for the products of acetolysis and, consequently, to the interpretation of the acetolysis reaction and the formula advanced for ouabain.

In conclusion we wish to acknowledge our indebtedness to Eli Lilly and Company and to Merck and Company for supplies of ouabain.

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## ON PROTEOLYTIC ENZYMES

### IX. THE INACTIVATION OF PAPAIN WITH IODINE

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(Received for publication, May 11, 1936)

When papain is treated with iodine, the activity of the enzyme against gelatin is known to be lost (1, 2). It is possible, however, to restore at least a part of the activity by treatment with HCN. It was recently found that papain splits not only proteins, but also a series of simple synthetic substrates such as hippurylamide, benzoylisoglutamine, and acylated peptides (3, 4). It was shown furthermore that the activity of papain against hippurylamide could be suppressed by iodine exactly as is its activity against gelatin. When the iodine-oxidized papain was subsequently treated with HCN, it became active against gelatin, but no activity against hippurylamide could be found. The experiments reported in this paper have been performed in order to ascertain whether the difference in the behavior of reactivated papain against various substrates is also to be found when the inactivation of papain is performed with the minimum amount of iodine.

At present, a papain preparation is at our disposal, which, before HCN activation, has an appreciably greater effect on gelatin than our earlier preparations had. Therefore, we investigated the effect of the new preparation on synthetic substrates. Benzoylisoglutamine was used in addition to hippurylamide for comparative purposes, since it is split faster by papain.

In Table I three papain solutions, which were obtained from two different solid papain preparations, are compared. Papains I and II were prepared from the same solid papain. The extraction of the solid material was accomplished with greater thorough-

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ness in the case of Papain II. Papain III is the preparation used in the experiments reported in Paper VIII of this series (5). After activation with HCN, its activity against gelatin is comparable with that of the present preparation; however, before HCN activation it is much less active than the present preparation.

TABLE I  
*Activation of Natural Papain*

Enzyme	Substrate	Hydrolysis in ml. of 0.01 N KOH					
		1 hr.	2 hrs	4 hrs	6 hrs.	18 hrs.	24 hrs
Natural Papain I	Gelatin	0.46					1.03
HCN-Papain I	"	0.73					1.49
Natural Papain I	Hippurylamide				0.04		0.09
HCN-Papain I	"				0.54		0.96
Natural Papain I	Benzoylisoglutamine		0.25				0.65
HCN-Papain I	"		0.62				0.93
Natural Papain II	Gelatin	0.63	0.84		1.06	1.20	1.19
HCN-Papain II	"	0.89	1.07		1.29	1.59	1.61
Natural Papain II	Hippurylamide			0.03			0.10
HCN-Papain II	"			0.55			0.95
Natural Papain III	Gelatin		0.36				0.85
HCN-Papain III	"		0.90				1.68
Natural Papain III	Hippurylamide		0.03				0.05
HCN-Papain III	"		0.21				1.06

In every experiment the solution contained for each ml. of volume 0.05 mM of synthetic substrate or 40 mg. of gelatin, 0.1 ml. of 0.02 N disodium citrate buffer (pH 5.0), and 0.2 ml. of enzyme solution. Benzoylisoglutamine was dissolved in the equivalent amount of 0.5 N sodium acetate. The temperature was 40°. The digestion was measured in ml. of 0.01 N KOH per 0.2 ml. of solution, according to the method of Grassmann and Heyde (6). 1 ml. represents 100 per cent splitting for all synthetic substrates.

Both preparations were obtained in Ceylon from the milky sap of *Carica papaya* by careful vacuum evaporation, and were kept in storage at a temperature of approximately 5°.

As may be seen from Table I, those preparations of natural papain which, even before HCN activation, have a very strong

effect on gelatin manifest also a distinct splitting of hippurylamide. But the extent of this splitting is, indeed, very small and hardly exceeds the limits of experimental error. The splitting of benzoylisoglutamine by natural papain is considerably faster. In

TABLE II  
*Activation of Papain after Oxidation with Iodine*

Substrate	En- zyme No.	Hydrolysis in ml. of 0.01 N KOH						
		1 hr.	2½ hrs.	5 hrs.	8 hrs.	24 hrs.	48 hrs.	120 hrs.
Gelatin	I	0.73				1.49		
	II	0.00				0.17		
	III	0.64				1.22		
Benzoylisoglutamine	I		0.62			0.93		
	II		0.02			0.10		
	III		0.50			0.67		
Hippurylamide	I			0.54		0.96		
	II			0.00		0.01		
	III			0.30		0.75		
Carbobenzoxylglutamylglycylglycine	I				0.44	0.86		
	II				0.00	0.02		
	III				0.16	0.43		
Diglycyl-L-leucylglycine	I						0.47	0.73
	II						0.00	0.00
	III						0.07	0.20
Triglycyl-L-leucylglycine	I					0.53		1.19
	II					0.00		0.00
	III					0.23		0.78

Enzyme I represents HCN-papain; Enzyme II, natural papain after iodine treatment; Enzyme III, natural papain after treatment with iodine and then with HCN. Considering the different concentrations of Enzymes I, II, and III, there was used for the individual experiments 0.2 ml. of Enzyme I, 0.25 ml. of Enzyme II, or 0.33 ml. of Enzyme III. Carbobenzoxylglutamylglycylglycine was dissolved in the equivalent amount of 0.1 N NaOH. The other details of these experiments are the same as those in Table I. (The preparation of carbobenzoxylglutamylglycylglycine will be described in a later communication.)

this case, one may attain a high splitting without difficulty and may also isolate the splitting products (benzoylglutamic acid and ammonia). These are the same products as those obtained with HCN-papain. The action of HCN on papain therefore effects

no change in specificity with benzoylisoglutamine, but does effect an increase in the amount of active enzyme and, accordingly, an acceleration of the splitting of the substrate.

In Table II experiments involving the influence of iodine on the papain digestion of a number of synthetic substrates are described. There are included two free polypeptides which have been found, through experiments carried on in cooperation with Dr. Joseph S. Fruton, to be hydrolyzed with papain. From Table II it may be seen that the inactivation of papain with iodine extends not only to the splitting of gelatin and acylated peptides, but also to the splitting of free peptides.

One finds reported in the literature that oxidized papain can be completely reactivated with reducing agents. In our experiments with iodine, this goal could not be reached. The inactivation was undertaken with the minimum amount of iodine, a condition under which the enzyme could be reactivated with HCN towards all the substrates investigated.<sup>1</sup> However, the regenerated activity was always less than that of the original papain after activation.<sup>2</sup> Although the same reactivated enzyme preparation was used in all cases, the loss in activity was more pronounced in the case of substrates with a smaller splitting rate. Apparently, this is due to the presence of inhibitive substances formed in consequence of the treatment with iodine.

Grateful acknowledgment is made to Dr. William F. Ross for assistance in preparing this manuscript.

#### EXPERIMENTAL

*Natural Papain*—225 mg. of powdered papain were shaken for 1 hour with 15 ml. of water; the solution was filtered, combined with 25 ml. of citrate buffer, pH 5.0, and diluted with water to 50 ml.

*HCN-Papain*—To a solution of 225 mg.<sup>3</sup> of papain in 15 ml. of water were added 25 ml. of citrate buffer, pH 5.0, and 10 ml. of

<sup>1</sup> It appears possible that the inactivation of papain by hydrogen peroxide is also reversible with regard to synthetic substrates, when performed with the minimum amount of hydrogen peroxide.

<sup>2</sup> Maschmann and Helmert (7) have found, in experiments with gelatin, that the inactivation of papain with oxygen in the presence of cysteine-heavy metal was only partially reversible.

1.2 per cent HCN. The solution was warmed to 40° for 2 hours before being used.

*Iodine-Inactivated Papain*—0.05 N iodine in aqueous KI was added dropwise, with shaking and cooling, to 40 ml. of natural papain (as above) until the first faint yellow coloration occurred. This point was reached after the addition of about 2 ml. After the oxidized solution had been allowed to stand another hour at room temperature, 1 ml. of 0.1 N NaOH was added and the solution made to 50 ml. with water.

*Papain-HCN Reactivated after Iodine Oxidation*—35 ml. of iodine-inactivated papain solution were combined with 15 ml. of 1.2 per cent HCN and maintained for 2 hours at 40° before use.

0.2 ml. of either of the first two enzyme solutions corresponds to 0.25 ml. of iodine-inactivated or 0.33 ml. of the reactivated enzyme solution.

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## ON PROTEOLYTIC ENZYMES

### X. THE ENZYMES OF PAPAIN AND THEIR ACTIVATION

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(Received for publication, May 11, 1936)

The botanist Vines believed that he was able to separate both peptone-forming and peptone-splitting enzymes from the milky sap of *Carica papaya* (1). Applying quantitative methods to the hydrocyanic acid activation of papain, Willstätter and Grassmann (2) concluded that papain is a homogeneous enzyme. They assumed that hydrocyanic acid plays the rôle of a kinase that extends the specificity range of the enzyme. According to them, papain converts proteins into peptones and is not able to attack the latter; on the other hand, HCN-papain digests both proteins and peptones. Grassmann later suggested (3) that papain alone is without action on proteins and peptones, and that it requires for the different stages of its activity different specific natural activators. More recent investigators (4, 5) have adopted the view that active papain is a sulfhydryl compound which is transformed reversibly into the inactive form by oxidation. This theory has not been extended to include an explanation of the specificity of the enzyme.

The experiments to be described in this paper were undertaken to compare the digestion of benzoylisoglutamine, a synthetic substrate (6), with that of peptone from albumin.<sup>1</sup> In the presence of phenylhydrazine the splitting of the two substrates by HCN-papain was effected quite differently. The hydrolysis of benzoylisoglutamine (Table I) was completely suppressed by modest additions of phenylhydrazine, but peptone digestion was not diminished. As a basis for comparison, the uninhibited enzyme in

<sup>1</sup> Merck's peptone *ex albumine* was employed; it should be similar to the preparation used by Willstätter and Grassmann.



varying concentrations was allowed to act upon the substrates. The results from these dilution experiments have been plotted in Fig. 1.

From the action of phenylhydrazine on HCN-papain it is possible to differentiate between two enzymes. One splits benzoylisoglutamine and hippurylamide and is completely suppressed by phenylhydrazine; it may be designated *Papain Pepti-*

TABLE I  
*Effect of Phenylhydrazine on HCN-Activated Papain*

C <sub>6</sub> H <sub>5</sub> ·N <sub>2</sub> H <sub>2</sub> , mm per ml. test solution	Gelatin			Peptone <i>ex albumine</i>			Benzoyliso- glutamine
	1 hr.	3 hrs.	24 hrs	1 hr.	3 hrs.	24 hrs.	1 hr
		1.18	1.70		0.31	0.64	
					0.43	0.77	
0.005		0.75	1.58		0.37	0.80	
0.005					0.39	0.89	
	1.08			0.26			0.37
0.0009	0.81			0.30			0.23
0.0019	0.73			0.26			0.11
0.0075	0.53			0.27			0.01
0.020	0.52						
0.030	0.44						
0.030	0.37			0.27			0.00
0.050	0.45						
0.056	0.42			0.25			0.02
0.080	0.42						

The increase is measured in ml. of 0.01 N KOH per 0.2 ml. of test solution. The concentration of HCN is constant, 0.018 mm per ml. of test solution. Hydrolysis equivalent to 1.0 ml. of alkali in the case of benzoylisoglutamine represents 100 per cent splitting.

*dase I.* The other enzyme splits albumin peptone and is not inhibited by phenylhydrazine; it is designated *Papain Peptidase II*. These names are only provisional and may be altered as soon as the specificity of the enzymes becomes known in detail.

Both Papain Peptidase I and Papain Peptidase II have a rôle in the splitting of gelatin. When small but varying portions of phenylhydrazine are added to samples of HCN-papain and these are used, after standing a while, for the digestion of gelatin, there

occurs an inhibition of the enzymic hydrolysis. Such experiments are included in Table I. It is clear that up to a certain concentration of phenylhydrazine the inhibition of gelatin hydrolysis is greater, the greater the amount of added phenylhydrazine. But when the phenylhydrazine in the test solution reaches a concentration of approximately 0.03 mm per ml. (0.32 per cent), a maximum inhibition is reached, and further increase in the phenylhydrazine concentration does not effect further inhibition. The digestion of gelatin by HCN-papain consists, therefore, of two

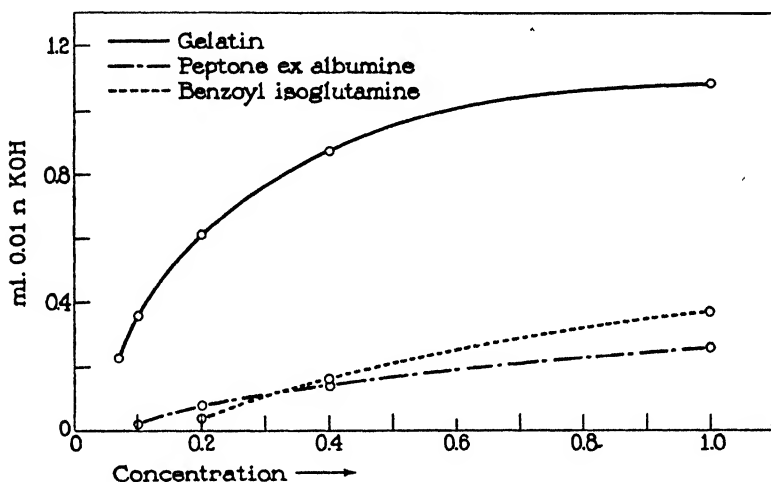


FIG. 1. Hydrolysis is represented by increase in titration per 0.2 ml. of splitting solution after 1 hour at 40°. A single HCN-papain solution (concentration equal to 1.0), of the usual preparation, was diluted as indicated.

different enzymic processes, one of which is inhibited by phenylhydrazine, the other not.

It should be stressed that HCN-papain still digests gelatin after maximum inhibition with phenylhydrazine. Thus, after complete inactivation of Papain Peptidase I, Papain Peptidase II, which remains, still splits gelatin. Papain Peptidase II is therefore not restricted to peptones but attacks gelatin as well.

Recently, the working hypothesis was suggested that Papain Peptidase I contains an aldehyde group (7); the inhibition by phenylhydrazine would thus be explained. We have tried to

bring additional experimental support to this theory. If the suppression by phenylhydrazine depends upon the conversion of a carbonyl group into its hydrazone derivative, one would expect (1) that hydroxylamine would also inhibit, (2) that a tertiary

TABLE II  
*Effect of Various Reagents on Papain*

Reagent, R	Mm per ml. solution		Gelatin			Peptone <i>ex albumine</i>			Benzoylisoglu- tamine		
	R	HCN	1 hr	3 hrs	24 hrs	1 hr	3 hrs	24 hrs	1 hr.	3 hrs.	24 hrs
HONH <sub>2</sub> *	0.030	0.018	0.30	0.38	0.47	0.05	0.16	0.34	0.02	0.0	0.0
C <sub>6</sub> H <sub>5</sub> N†	0.030	0.018	1.17	1.50	1.79	0.24	0.43	0.68	0.38	0.67	0.95
		0.018							0.66	0.92	0.97
									0.66	0.94	1.12
C <sub>6</sub> H <sub>5</sub> ·N <sub>2</sub> H <sub>3</sub>	0.0015	0.018		1.05	1.72	0.42	0.84		0.05	0.11	
C <sub>6</sub> H <sub>5</sub> ·N <sub>2</sub> H <sub>3</sub> ‡	0.0015								0.07	0.06	
+		0.018		1.30	1.96	0.26	0.58	0.19	0.46	1.09	
C <sub>6</sub> H <sub>5</sub> CHO	0.004										
C <sub>6</sub> H <sub>5</sub> CHO	0.004	0.018		1.28	1.90	0.34	0.69	0.66	0.95	1.04	
C <sub>6</sub> H <sub>5</sub> ·N <sub>2</sub> H <sub>3</sub>	0.015	0.018							0.0	0.0	0.02
C <sub>6</sub> H <sub>5</sub> ·N <sub>2</sub> H <sub>3</sub> ‡	0.015										
+		0.018	0.85	1.18	1.89	0.18	0.31	0.57	0.05	0.11	0.41
C <sub>6</sub> H <sub>5</sub> CHO	0.030										
C <sub>6</sub> H <sub>5</sub> CHO	0.030	0.018	1.09	1.47		0.34	0.40		0.24	0.56	

The hydrolysis is measured as increase in ml. of 0.01 N KOH per 0.2 ml. of test solution. Temperature, 40°.

\* HONH<sub>2</sub>·HCl was weighed out, treated with a molecular equivalent of 1 N NaOH, and added to the usual HCN-papain preparation; the enzyme was tested after 2 hours at room temperature.

† Introduced into the usual HCN-papain 1 hour before testing.

‡ Freshly distilled benzaldehyde added directly to the C<sub>6</sub>H<sub>5</sub>·N<sub>2</sub>H<sub>3</sub>-HCN-papain enzyme preceding. Hydrazone formation occurred at once. After 3 hours the latter was centrifuged off and the resulting enzyme tested.

amine like pyridine would not inhibit, and (3) that the inhibition of phenylhydrazine would be removed by the addition of simple aldehydes, analogous to the splitting of sugar hydrazones by benzaldehyde. Each of these three conclusions has actually been

substantiated experimentally. These data are presented in Table II. In the case of hydroxylamine, there seems to be an effect on the hydrolysis of gelatin and peptone in addition to the suppression of Papain Peptidase I.

Still another way was found to differentiate between Papain Peptidase I and Papain Peptidase II. A normal HCN-papain preparation was treated with so much iodine that no digestion of gelatin occurred within 24 hours. This solution was then maintained at 40° and after 2 days the ability to digest gelatin and

TABLE III  
*Spontaneous Reactivation of Iodine-Treated Papain*

Iodine, mm per ml. test solution	Hrs at 40° after addition of iodine	Gelatin				Peptone <i>ex albumine</i>		Benzoylisoglu- tamine	
		3 hrs.	24 hrs.	50 hrs.	96 hrs.	3 hrs.	24 hrs.	3 hrs.	24 hrs.
0.001	1	0.03	0.07	1.41	2.00				
	50	0.03	1.23						
	150	1.13	1.70						
0.001	1*	0.02	0.04	1.67					
	50*	0.08	1.71	1.88					
0.001	120	0.97	1.60			0.32	0.64	0.0	0.01
	300							0.0	0.0

To 5 ml. of HCN-papain was added, slowly and with shaking, 1 ml. of 0.05 N iodine in aqueous KI solution. The resulting solution was maintained at 40°, samples being removed at the hours indicated for testing. Hydrolysis is measured in ml. of 0.01 N KOH per 0.2 ml. of test solution. The concentration of HCN is 0.018 mm per ml. of test solution.

\* In the presence of a slight excess of toluene.

peptone began gradually to reappear (Table III). The ability to digest benzoylisoglutamine had not reappeared after 12 days.

This reactivation process is of interest not only because it demonstrates the presence of two enzymic constituents of HCN-papain, but also because it is a reversible inactivation of Papain Peptidase II, the reversion of which is not effected by hydrocyanic acid even in the presence of a large excess of this reagent, and which therefore does not consist of a simple transformation of a sulfhydryl into a disulfide group. This is true also of the inactivation of Papain Peptidase I by phenylhydrazine,

which cannot be reversed by hydrocyanic acid but can be reversed by benzaldehyde. There are groups, other than those of the  $-\text{SH} \rightleftharpoons -\text{S}-\text{S}-$  system, having a decisive influence on the activity of the papain constituents.

Papain has previously been regarded as a typical proteinase. Now that means of distinguishing two papain constituents are available, it is necessary to consider whether both of these constituents are to be classified as proteinases. This question is of special interest in the case of Papain Peptidase I, since it splits synthetic substrates of a low molecular weight and since its specificity is fairly well known.

At present, the proteolytic enzymes are divided into two main groups. The *proteinases* are those enzymes which are capable of splitting genuine proteins, but which are ineffective against lower degradation products and those peptides previously synthesized. The *peptidases* (dipeptidase, aminopeptidase, and carboxypeptidase), according to definition, split exclusively the low intermediary products of protein degradation and also synthetic peptides, but are never effective against proteins (8, 9).

This classification is based upon the chain length of the substrate<sup>2</sup> and leads to difficulties in the case of Papain Peptidase I. Although this enzyme is capable of splitting synthetic substrates of low molecular weight, there is no evidence that it is restricted to such substrates. According to the above definition, Papain Peptidase I would not be a proteinase but would belong to the same group as dipeptidase, aminopeptidase, and carboxypeptidase. In its specificity, however, it differs fundamentally from these peptidases, and this difference offers a basis for modifying the present classification of proteolytic enzymes.

Aminopeptidase, carboxypeptidase, and dipeptidase have the same specificity requirements in that the action of each is directed to the end of the peptide chain of the substrate. These peptidases require either a terminal amino or a terminal carboxyl group, or both, and split only terminal peptide linkages. The same is true, as far as is known, for several special enzymes such as prolinase (10, 11) and protaminase (12). Papain Peptidase I is therefore the first proteolytic enzyme of known specificity which requires

<sup>2</sup> Actually only dipeptidase is known to require a substrate of a prescribed chain length.

neither terminal amino nor carboxyl groups and which is not restricted to the splitting of terminal peptide linkages. It is influenced by the nature of the amino acid residues forming or lying near the peptide linkage which is split (6, 13).

It is therefore possible to classify the two types of peptidases as *exo*peptidases, which are restricted to terminal peptide linkages, and *endo*peptidases, which are not thus restricted (14). The *exo*peptidases, such as aminopeptidase and carboxypeptidase, should have a very restricted action on the gigantic protein molecules, because these have so few terminal groups. The *endo*peptidases, on the other hand, should be more suited for digesting proteins, and it is to be expected that the proteinases will fall largely in this group when suitable known substrates are found.

Clupean, a polypeptide of thirteen amino acids obtained from clupein, has recently been shown by Waldschmidt-Leitz and Kofranyi to be split at four points within the peptide chain by active trypsin, and at two by chymotrypsin (15). This fact has led these authors to the decision that the molecular weight is no longer the deciding factor between proteinase and peptidase activity. It also indicates that trypsin and chymotrypsin are true *endo*peptidases, as defined in this paper.

As there are two distinct enzymes present in HCN-activated papain, it was desirable to determine to what extent their presence is manifested in natural papain before HCN treatment. The digestion of albumin peptone by papain, in the absence of hydrocyanic acid and of phenylhydrazine, is small and attains well defined values only after several hours (Table IV). Under the same conditions the digestion of benzoylisoglutamine is appreciable. By the addition of phenylhydrazine the splitting of benzoylisoglutamine was almost completely suppressed, but the digestion of albumin peptone was increased several fold. The latter effect may be interpreted either as the direct activation of Papain Peptidase II by phenylhydrazine, or as an indirect activation through the removal of a naturally present inhibitor.

The activation of Papain Peptidase II, which occurs when phenylhydrazine is added to natural papain, is coincident with the inactivation of Papain Peptidase I. In fact, the two phenomena seem to be interdependent. Thus, the activation of papain cannot be a simple reduction process. The authors wish to suggest the

hypothesis that in natural papain there exists a compound between Papain Peptidase I and Papain Peptidase II, in which the activities of these peptidases are inhibited. This compound is in equilibrium with the two free peptidases. The activation of natural papain is thus chiefly the dissociation of this compound caused by the addition of an activator to one of the peptidases, thus upsetting the equilibrium. Actually, many papain activators, such as  $H_2S$ ,  $HCN$ ,  $-SH$  compounds, and sulfites, may react with aldehyde groups, and it has been pointed out that an aldehyde group may be present in Papain Peptidase I. The reversible inactivation of active papain may, in accord with this theory, be the simple

TABLE IV  
*Action of Phenylhydrazine on Natural Papain*

$C_6H_5N_2H_3$ mm per ml. test solution	Gelatin			Peptone <i>ex albumine</i>			Benzoylisoglutamine		
	1 hr	3 hrs	24 hrs	1 hr.	3 hrs	24 hrs	1 hr.	3 hrs.	24 hrs.
	0 43	1 05 0 93	1 52 1.34	0 06	0.08 0.06 0.10	0.16 0.11 0.15		0.62 0.47	1.01 0.94
0 01	0.56 0.33	0.84 0.64	1.19	0.06 0.09	0 10 0 21	0.10 0.50	0.01	0 04	0.05
0.01	0 27	0.49		0 07	0 19	0.50			
0.03	0 35	0.63	1.25	0 12	0 29	0 47	0.03	0.05	0.09

The hydrolysis is measured as increase in ml. of 0.01 N KOH per 0.2 ml. of test solution.

removal of essential activators—by oxidation, complex formation, etc. (*cf.* (16) and (17)). It is planned to test this working hypothesis with additional experiments, and to compare the behavior of other proteinases with the Papain Peptidases I and II.

#### EXPERIMENTAL

##### *General Procedure for Enzyme Test Solutions*

The composition of the test solutions was maintained the same in all experiments. Each ml. contained 40 mg. of water-free gelatin, 47 mg. of peptone or 0.05 mm of synthetic substrate, 0.10 ml. of disodium citrate buffer, pH 5.0 (in addition to that in the enzyme solution), and 0.2 ml. of enzyme solution or its equivalent.

(Benzoylisoglutamine was dissolved with an equimolecular amount of 0.5 N sodium acetate.) 0.2 ml. samples of this solution were titrated with 90 per cent alcoholic 0.01 N KOH with thymolphthalein. An increase of 1.0 ml. of base represents 100 per cent splitting of synthetic substrate. Digestion was at pH 5.0 and at 40°.

### Enzyme Preparation

Our papain was the residue obtained by vacuum evaporation of the sap of green fruit of *Carica papaya*.

*Natural Papain*—225 mg. of finely powdered papain were shaken for 1 hour with 15 ml. of water, filtered, combined with 25 ml. of disodium citrate buffer, pH 5.0, and the solution made to 50 ml. with water.

*HCN-Papain*—To a 15 ml. solution from 225 mg. of papain were added 25 ml. of citrate buffer and 10 ml. of 1.2 per cent HCN; the solution was kept for 2 hours at 40° before use.

*Phenylhydrazine-Papain*—To the normal HCN-papain or natural papain solution was added the required amount of a freshly prepared 10 per cent aqueous phenylhydrazine solution. It was allowed to stand for 1 hour at room temperature before use.

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## A FAT OXIDATION SYSTEM IN LUPINUS ALBUS\*

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It has been observed that pulverized seeds of *Lupinus* will consume oxygen and produce carbon dioxide when shaken with water in the Warburg respirometer.<sup>1</sup> In view of the results that have been obtained with such material as zymase and ground sea urchin eggs, the investigation of this phenomenon may be expected to contribute to our knowledge of biological oxidations. The experiments to be reported are concerned with the isolation of the reactants from the seed powder, and with the description of the process in terms suitable for comparison with known oxidation systems. It has been possible to trace the activity of the powder to two components obtainable in stable form. One of these, the petroleum ether extract of the seeds, is the substrate; the other, a substance precipitated from the water extract by half saturation with ammonium sulfate, is a mixture of enzymes. That several types of catalysis are involved is concluded from a study of the effects on the system of temperature, carbon monoxide, potassium cyanide, urethane, and phosphate buffer. In general, the results are of interest in relation to cyanide-stable respiration, fat metabolism, and the theory of temperature characteristics.

The material used consisted of three products designated *powder*, *oil*, and *enzyme* derived from seeds<sup>2</sup> of a strain of *Lupinus albus*, L.

\* The material in this paper was presented at a meeting of the New England Section of the American Society of Plant Physiologists at Durham, New Hampshire, May 18, 1935, under the title, "Respiratory systems of *Lupinus albus*."

<sup>1</sup> Unpublished work of Dr. A. E. Navez in this laboratory in 1929. Also he observed the phenomenon in *Pisum* and *Phaseolus*, but not in *Triticum*, *Hordeum*, or *Avena*.

<sup>2</sup> Very kindly supplied by Dr. Navez.

inbred for 3 years. Of these products, the *powder* was prepared from seeds from which the testa and embryo had been removed. The seeds were ground in a porcelain mortar until the resulting meal was fine enough to pass a screen of bolting silk of 23 threads to the cm. Further grinding was undesirable because of loss of activity in the product, as has also been reported by Harden and McFarlane (1930) in the preparation of zymase. The powder, kept in a stoppered bottle at room temperature, is quite stable and one preparation has retained its activity undiminished for 3 years. The oil and enzyme were obtained from seeds ground to a coarse meal in a hand mill. This meal was first extracted in a Soxhlet apparatus with petroleum ether having a boiling point of from 20–30°, and then with 10 per cent NaCl solution. The petroleum ether extract, freed from its solvent by vacuum distillation at room temperature, is a yellow-brown viscous liquid called the *oil*. In one determination this amounted to 8.8 per cent of a 25 gm. portion of meal, which agrees with the 8.88 per cent reported by Guillaume (1923). Its iodine number was found to be 104. The brine extract was half saturated with ammonium sulfate and the resulting bulky, white precipitate separated from the supernatant liquid by centrifuging. This precipitate, called the *enzyme*, was dried on filter paper in air at room temperature and then ground to a powder. Considerable salt was occluded in the precipitate, so that it was readily soluble in distilled water.

Oxygen consumption and carbon dioxide production were measured manometrically with Warburg microrespirometers; a description of the apparatus and the theory of the method are given by Dixon (1934). The standard reaction mixture was a suspension of 100 mg. of powder in 1.0 ml. of water. The powder was placed at the bottom of the conical Warburg vessel, about 18 ml. in volume, and the water in the side arm; in the inset was 0.2 ml. of 10 per cent NaOH. When oil was to be part of the reaction mixture, the vessel was used as a weighing bottle, the oil being pipetted in before the powder was added, so that the two did not mix until the experiment had been started. The vessel, attached to the manometer, was placed in a thermostat of the type described by Stier and Crozier (1932–33), the temperature being maintained to within  $\pm 0.05^\circ$ . After a thermal adaptation period of 15 minutes, the water was poured onto the powder from

the side arm. The first reading was then taken and the shaker started immediately. The respirometers were shaken at a rate of 110 oscillations per minute through 9.5 cm. Although oxygen consumption is dependent upon shaker speed in this range, a higher rate could not be used. Readings were continued at intervals, depending upon the rate of oxygen consumption, until the reaction was complete, or from 3 to 12 hours, according to the temperature. When carbon dioxide was to be measured, a vessel containing the same reaction mixture was run without alkali in the inset.

With this technique, measurements of oxygen consumption were reproducible to within 5 per cent, as is shown by the results

TABLE I

*Total Oxygen Consumption of Standard Reaction Mixtures at 25° in C.Mm.*

Experiment No.	Vessel No.			
	1	2	3	4
104	175	155	160	152
106	175	155	156	154
107	169	170	165	165
108	173	160	153	162
Mean.....	.162			
Average deviation.....	6.9			
Deviation, %..	4.3			

of a series of four runs with four vessels, under conditions as constant as possible (Table I). The precision for carbon dioxide measurements was necessarily less. The quotients of total carbon dioxide produced to oxygen consumed in six comparable experiments were 0.38, 0.29, 0.31, 0.29, 0.34, and 0.29, for which the average deviation is about 10 per cent.

#### *General Properties of the System*

1. Oxygen is consumed and carbon dioxide is produced at rates that are greatest at the beginning of the reaction and gradually fall to zero in a manner approaching that of a first order reaction. Data are given in Table VI and curves in Figs. 1, 2, 3, 8, and 9.

2. The ratio of the total amounts of carbon dioxide produced to

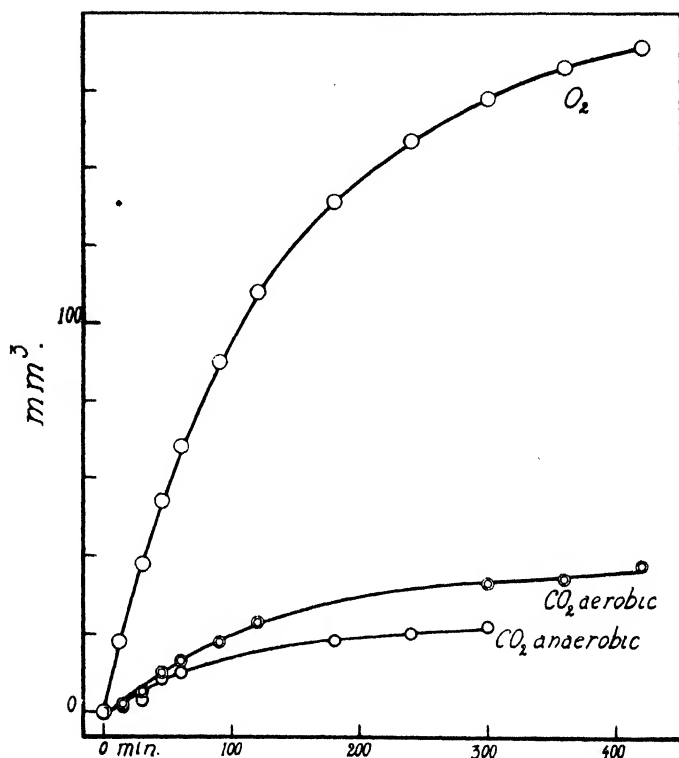


FIG. 1. Curves of oxygen consumption, carbon dioxide production, and anaerobic carbon dioxide production at 15° for standard reaction mixtures.

TABLE II

Effect of Water Concentration on Total Oxygen Consumption of 100 Mg. of Powder at 20°

Experiment No.	Volume of water added	O <sub>2</sub> consumed
	ml.	c.mm.
52.2	0.2	9
56.2	0.5	46
50.2	1.0	194

oxygen consumed in a completed reaction will be referred to as the CO<sub>2</sub>/O<sub>2</sub> quotient; this varies from about 0.33 to 0.20.

3. When more than 5 per cent by volume of oxygen is present

in the atmosphere in the Warburg vessel, the system is independent of oxygen tension. Oxygen consumption ceases when the vessel is filled with pure nitrogen, but there is some carbon dioxide produced anaerobically. Comparable curves for oxygen consumption

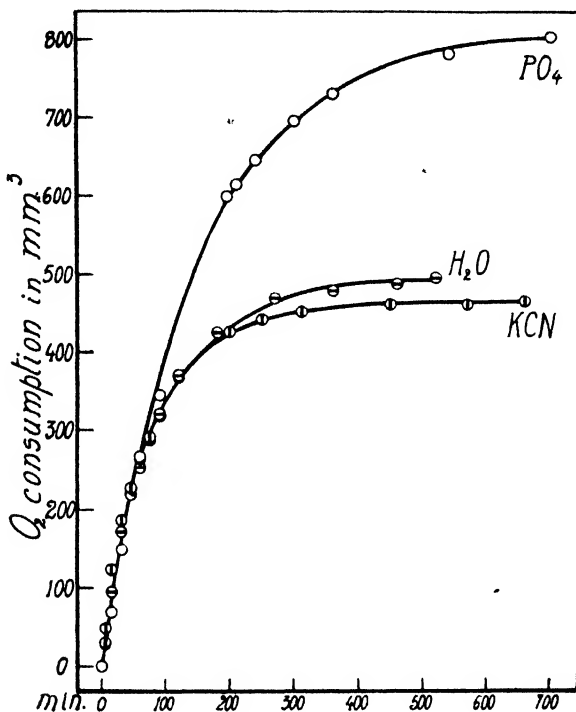


FIG. 2. Effects of 0.001 M KCN and phosphate buffer on oxygen consumption by reaction mixtures of 100 mg. of powder, 46 mg. of oil, and 1.0 ml. of solution. The buffer solution consisted of 8 parts of  $m/15$   $\text{Na}_2\text{HPO}_4$  and 2 parts of  $m/15$   $\text{KH}_2\text{PO}_4$ , and has a pH of about 7.3. The temperature was  $25^\circ$ .

tion, carbon dioxide production, and anaerobic carbon dioxide production are given in Fig. 1.

4. The amount of water in the suspension determines to a certain extent the total oxygen consumption of the system, as may be seen from Table II. Fig. 6 also bears on this point.

5. The reaction has an optimum pH of about 7.0 in phosphate

buffer; almost no oxygen is consumed below pH 4.0 in HCl-citrate buffer or above pH 12.0 in NaOH-glycocoll buffer.

6. Acid is produced in small quantities during the reaction; in one case, the pH fell from 6.5 to 6.0 in the unbuffered system.

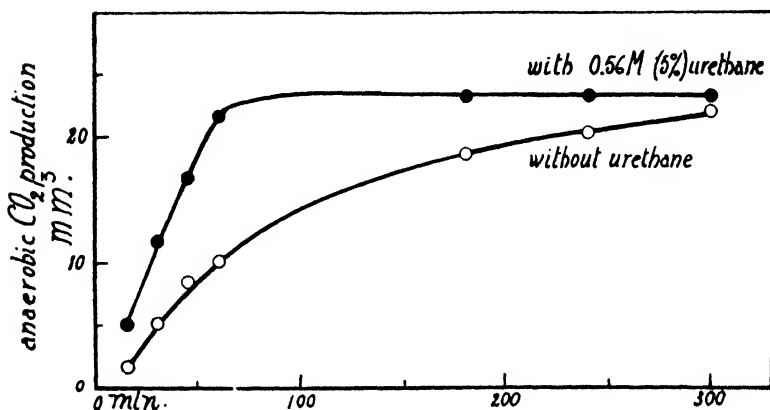


FIG. 3. The effect of 5 per cent urethane on anaerobic carbon dioxide production by standard reaction mixtures at 15°. The initial rate is increased, but the total amount produced is not affected under these conditions.

TABLE III

*Effect of 5 Per Cent Urethane on Oxygen Consumption and Carbon Dioxide Production*

Standard reaction mixture; temperature, 15°; time, 5 hours.

Experiment No.	Solution	O <sub>2</sub> consumed	CO <sub>2</sub> produced	R. Q.
		<i>c. mm.</i>	<i>c. mm.</i>	
125.1	Water	158		
132.1	"		35	0.22
129.6	5% urethane	84		
132.2	5% "		52	0.62

7. The total oxygen consumption of the system is increased by phosphate buffer, as is shown in Fig. 2. This increase was about 50 per cent at 25° and 100 per cent at 45°. The CO<sub>2</sub>/O<sub>2</sub> quotient was not changed by phosphate.

8. Oxygen consumption was inhibited about 50 per cent by 0.56 M (5 per cent) urethane and 80 per cent by a 1.0 M solution.

Phenylurethane inhibited 28 per cent at 0.001 M and 90 per cent at 0.005 M. Indications that carbon dioxide production is accelerated under anaerobic conditions and increased in amount in air are given in Fig. 3 and Table III.

9. Oxygen consumption is inhibited by carbon monoxide and the effect is not reversed by illumination (two 150 watt Mazda lamps at 10 cm.). With a CO/O<sub>2</sub> ratio of 15, an inhibition of 80 per cent was obtained. The carbon monoxide was led from the generator into the mixing tank without washing.

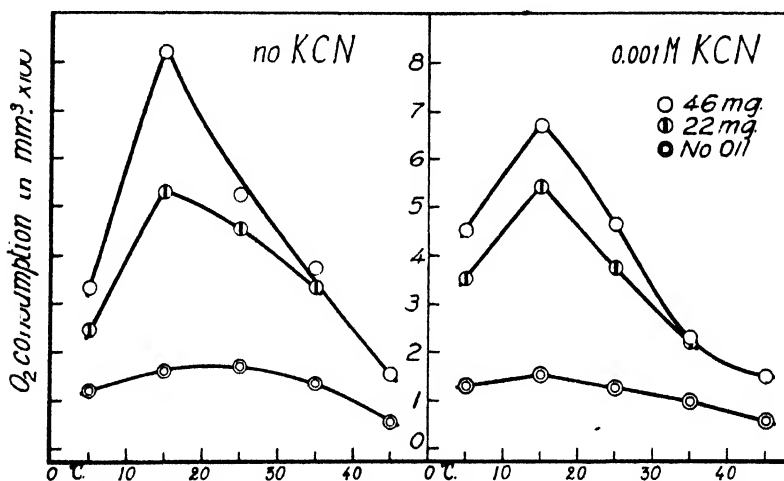


FIG. 4. The variation with temperature of the total oxygen consumption of standard reaction mixtures with and without added oil, and in the presence and absence of 0.001 M KCN.

10. The action of 0.001 M KCN may be summarized as follows: (a) With no added oil there is no effect at 5°, 15°, and 45° and an inhibition of about 30 per cent at 25° and 35°. (b) With added oil, there is an increase in oxygen consumption of 40 per cent at 5° and an inhibition of 40 per cent at 35°. There is no effect at 45° and about 20 per cent inhibition at 15° and 25°.

The data appear in Fig. 4. Cyanide does not affect the CO<sub>2</sub>/O<sub>2</sub> quotient.

11. Fig. 4 also shows the variation of total oxygen consumption with temperature. It is remarkable that while total oxygen con-



sumption decreases with rise in temperature, the rate of the reaction increases, as may be seen from Figs. 10 and 11. At 50° there is no appreciable oxygen consumption.

*Components of the System*

The oxidation system in *Lupinus* powder is easily separated into two fractions. When a freshly prepared suspension of powder in water was centrifuged, a slightly cloudy, pale yellow centrifugate and a closely packed, mealy precipitate were obtained. The centrifugate did not consume oxygen alone, nor did a suspension of the precipitate after it had been washed five times with water by centrifuging. When the two fractions were mixed, oxygen

TABLE IV  
*Reactions of Powder-Oil System*

Experiment No.	Temperature	Solution	Total O <sub>2</sub> consumed	Total CO <sub>2</sub> produced
	°C.		c.mm.	c.mm.
163.5	5	0.001 M KCN	43	
155.1	15	0.001 " "	275	
162.1	25	Water	145	
162.2	25	Phosphate buffer, pH 8.3	303	
162.3	25	0.001 M KCN	158	27
162.5	25	5% urethane	36	
164.5	45	0.001 M KCN	11	

In Experiment 162.3, the quotient is 0.17.

consumption was resumed. The active elements of the two fractions are the oil and the enzyme, whose preparation has already been described. The residue of the meal which has been extracted with both petroleum ether and salt solution is not necessary for the reaction, nor is the yellow pigment which is easily extracted with alcohol.

With a reaction mixture of 31 mg. of oil, 50 mg. of enzyme, and 1.0 ml. of water it was possible to duplicate qualitatively the results obtained with the powder-water mixture with regard to temperature, phosphate, urethane, cyanide, and the CO<sub>2</sub>/O<sub>2</sub> quotient. The data are given in Table IV. The amount of oxygen consumed in the powder-water reaction is proportional to the concentration of each component, as may be seen from Figs.

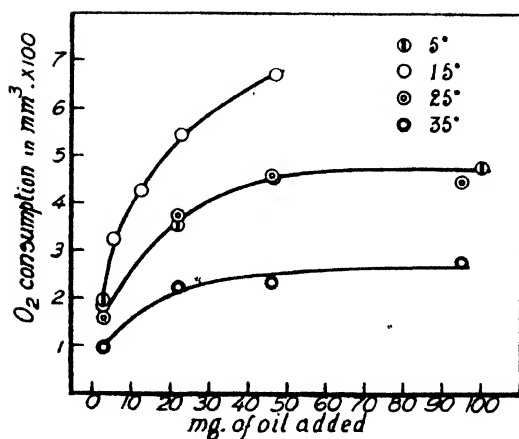


FIG. 5. The relation between total oxygen consumption and the amount of oil present, when the enzyme concentration is constant. The amounts of oil indicated are added to standard reaction mixtures.

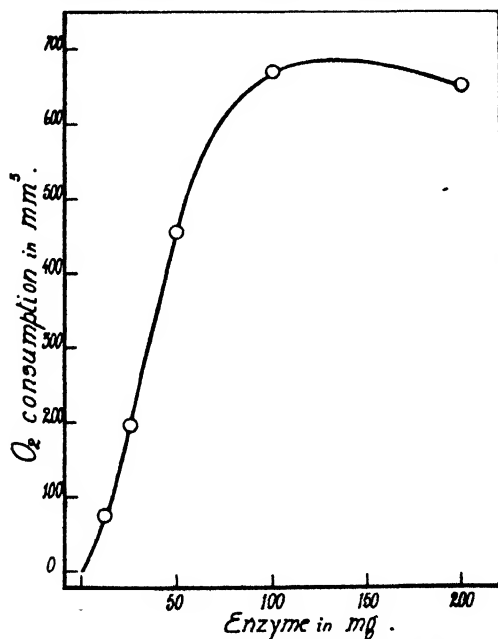


FIG. 6. Total oxygen consumption as a function of enzyme concentration. Various amounts of oil were added to the powder, so that the sum of oil in powder and added oil was 56 mg. in each case.

5 and 6. When the concentration is high enough, the system becomes saturated by either component.

The curve of oxygen consumption with time indicates that some component of the system is being exhausted or inactivated during the reaction. That this is not the oil is shown by the following experiment. A number of reaction mixtures in which oxygen consumption had gone to completion were collected and by centrifuging separated into the two components, these being called used centrifugate and used precipitate. A freshly prepared suspension of powder in water of corresponding volume and density was also centrifuged, yielding fresh centrifugate and fresh precipitate. Two reaction mixtures were made up consisting of (1) used precipitate and fresh centrifugate and (2) fresh precipitate and used centrifugate. The oxygen consumption of both was

TABLE V  
*Oxygen Consumption in Different Reaction Mixtures*

Experiment No	Reaction mixture	O <sub>2</sub> consumed in 3 hrs.
		<i>c.mm.</i>
115.3	Used ppt. and fresh centrifugate	77
115.4	Fresh " " used "	10
115.2	" " " fresh "	62
106.2	100 mg. powder and 1.0 ml. water	117

measured in the usual way. It was found that oxygen was consumed only by the mixture containing fresh centrifugate. The results are given in Table V.

Although the active element of the oil has not been identified, there are good indications as to its nature. (1) The CO<sub>2</sub>/O<sub>2</sub> quotient is low. The average of the six figures given above is 0.32 and the quotient for the two upper curves in Fig. 1 is 0.22; that for the enzyme-oil experiment given in Table IV is 0.17. From the data on ratio of the *rates* at different times during the reaction (Table VI), it is seen that carbon dioxide production proceeds at a rate independent of that of oxygen consumption. (2) The active element is soluble in both acetone and petroleum ether, but not in water or alcohol, and is stable at 60°. Extraction of the seeds with acetone at that temperature yielded an oil of about the same

activity as that obtained with petroleum ether. (3) Linseed oil and castor oil also have the property of consuming oxygen in the presence of the *Lupinus* enzyme preparation. Data are given in Table VII.

TABLE VI

*Data for Typical Experiment on Oxygen Consumption and Carbon Dioxide Production*

*Experiment 150.3.4*—Temperature 15°. Reaction mixture: 100 mg. of powder, 47 mg. of oil, 1.0 ml. of 0.001 M KCN.

Time	Vessel 3 0.3 ml. 10 per cent NaOH in inset $k_{O_2} = 1.69$		Vessel 4 No NaOH $k_{O_2} = 1.69; k_{CO_2} = 1.79$			Rate $CO_2$ Rate $O_2$ in terms of tangents to curves of $x$ vs. time
	$h_{O_2}$	$x_{O_2}$	$h_{(O_2 + CO_2)}$	$h_{CO_2}$	$x_{CO_2}$	
min.	mm.	c.mm.	mm.	mm.	c.mm.	
0	0	0	0	0	0	
6	20	34	12	8	14	0.29
15	51	86	34	17	30	0.31
30	94	159	74	20	36	0.40
45	134	226	92	42	75	0.36
60	164	277	112	52	93	0.30
75	187	316	130	57	102	0.26
90	206	348	145	61	109	0.19
120	239	403	173	66	118	0.16
180	290	490	216	74	133	0.14
250	325	549	249	76	136	0.09
365	360	608	282	78	140	0.07
420	369	623	291	78	140	
540	383	647				
690	392	662				

The quotient of the total gas exchange is 0.21. The first order velocity constants, determined graphically, are 0.0070 for oxygen consumption and 0.0167 for carbon dioxide production.

$k$  = vessel constant;  $h$  = corrected manometer readings;  $x$  = volume of gas change.

With regard to the nature of the enzyme component of the system, it is known that it is a mixture of at least two active elements and that it has the solubilities of a globulin. The bulk of the protein of these seeds is a globulin, conglutin, and a simplification of the method for its purification described by Osborne and Campbell (1897) has been used in the preparation of the enzyme. The

behavior of the system with cyanide is difficult to explain unless there are at least two enzymes involved. Further evidence for the complex nature of this component is provided by a heat inactivation experiment.

A solution of enzyme containing 50 mg. per ml. was exposed to a temperature of 45° in a water bath. At intervals, 1 ml. was removed to a vessel containing 31 mg. of oil. The oxygen consumption of the mixture was then measured at 15°. Although only five points were determined, it is clear that about two-thirds of the activity of the preparation was destroyed by exposure at 45°. When the enzyme was heated for 30 and 60 minutes, the amount of oxygen consumed was progressively much less, but only one curve can be fitted to the data for oxygen consumption after 1, 2,

TABLE VII  
*Susceptibility of Various Oils to Oxidation*

Total oxygen consumption of 31 mg. of oil, 50 mg. of enzyme, and 1.0 ml. of water.

Experiment No.	Temperature	Oil	O <sub>2</sub> consumed
			c.mm.
162.1	25	<i>Lupinus</i> petroleum ether extract	145
162.5-a	25	" acetone extract	178
157.5	25	Castor oil	57
169.5	45	Linseed oil	78

and 3 hours exposure. The integral curves of oxygen consumption against time are given in Fig. 7.

Since it has been found that other oils may be substituted for the petroleum ether extract of *Lupinus* seeds in this system, it is interesting to replace the enzyme component by a better known catalyst. The catalytic effect of iron combined in ferricyanide (Litarczec, 1928; Chow and Kamerling, 1934), in hemoglobin (Robinson, 1924), and in hemin pyridine (Kuhn and Meyer, 1929) on the oxidation of fats and fatty acids is well known. Accordingly, the oxygen consumption of 46 mg. of oil in 1.0 ml. of a solution containing 0.087 mg. of hemin<sup>3</sup> as hemin pyridine was measured at 35°. Oxygen is consumed at a rate of the same order

<sup>3</sup> The writer is indebted to Professor Louis Fieser of the Division of Chemistry for the gift of 10 mg. of hemin.

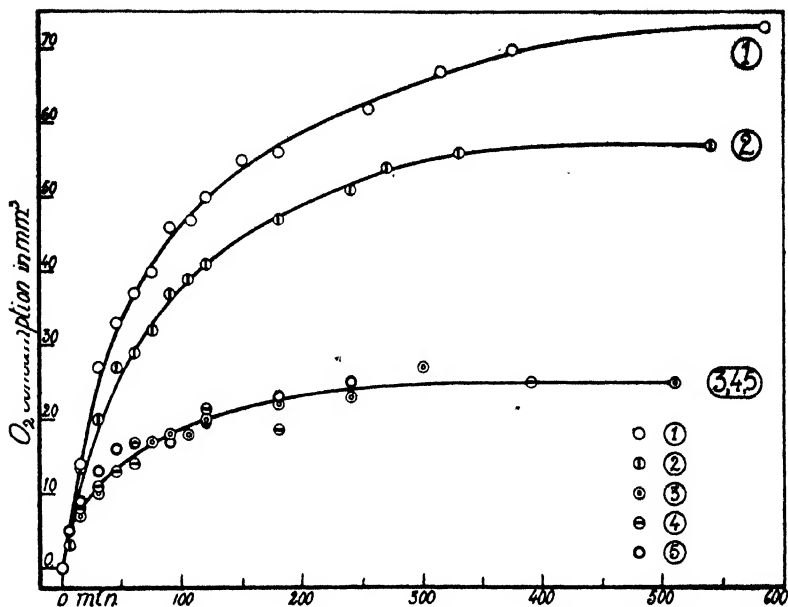


FIG. 7. Oxygen consumption by the enzyme-oil system at 15°. These curves show the effect of exposure of the enzyme at 45°, as described in the text. The symbols 1, 2, 3, 4, and 5 refer to the lengths of time the enzyme is heated; these are 0,  $\frac{1}{2}$ , 1, 2, and 3 hours, respectively.

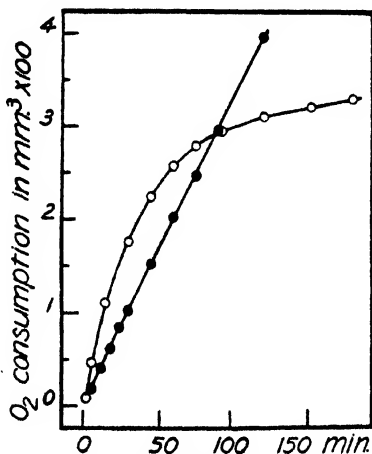


FIG. 8. Comparison of the catalytic effects of powder and hemin pyridine on the oxidation of 46 mg. of oil at 35°. The open circles represent powder and solid circles, hemin pyridine.

as the initial rate for a reaction mixture of 46 mg. of oil and 100 mg. of powder in 1.0 ml. of water. The data are given in Fig. 8.

Iron determinations made by the colorimetric method of Kennedy (1927) show that the powder contains from 0.0065 to 0.0086 per cent. The iron content of the enzyme is 2 or 3 times that of the powder, the result of one analysis being 0.023 per cent; no iron is found in the oil by this method. Using these data and calculating the iron in the hemin from the formula  $C_{34}H_{30}N_4O_4Fe$ , we see that in the experiment described in Fig. 8 there was 0.0086 to 0.0065 mg. in the powder and about 0.008 mg. in the hemin pyridine. In view of the similarity of the initial rates, this coincidence is striking.

#### *Temperature Characteristics for Oxygen Consumption*

In order to consider the velocity of the process as a function of temperature, it is necessary to describe the constantly changing rate by a single term, and for this the first order velocity constant has been found suitable. On the assumption that the total oxygen consumed is proportional to the amount of material that has been oxidized, the velocity constant for the process is given by the equation

$$k_1 = \frac{2.3 \log \frac{A - x_1}{A - x_2}}{t_2 - t_1}$$

in which  $x$  is the oxygen consumed at time  $t$  from the beginning of the reaction and  $A$  is the total amount of oxygen consumed. Although oxygen consumption by the system often approximates a first order reaction, good fits over the entire range of temperature were not obtained unless the reaction mixture contained KCN at 0.001 M. The effect of cyanide on the velocity constant is illustrated in Fig. 9, in which it is seen that in the absence of cyanide the values fall off badly at the end of the reaction. In this way velocity constants for oxygen consumption were determined in a series of experiments with and without added oil in the range from 5–45°. These data are plotted according to the equation

$$\text{Velocity} = ke^{-\mu/RT}$$

and it may be seen from Figs. 10 and 11 that  $\mu$ , the temperature characteristic, is a constant in the absence of added oil above 30°

and below  $20^{\circ}$ , and in the presence of added oil from  $5$ – $35^{\circ}$ , the values being 15,200, 3500, and 11,700 calories respectively.

From the data that have been presented, it is evident that the substance which consumes oxygen and produces carbon dioxide in the *Lupinus* oxidation system has the nature of a fat or fatty acid.

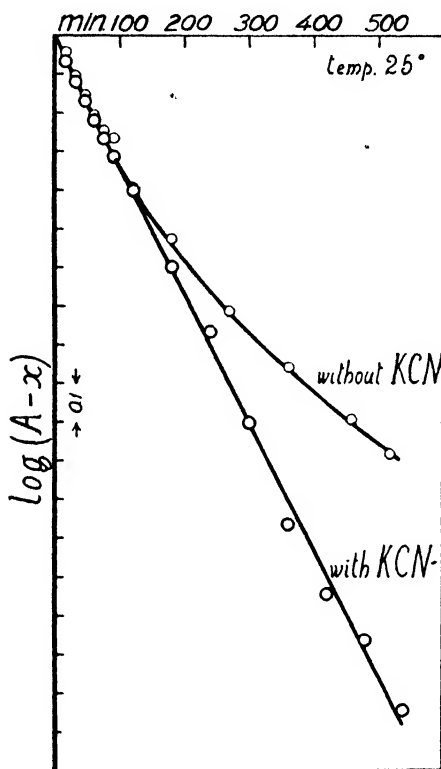


FIG. 9. Data for oxygen consumption of standard reaction mixtures with and without  $0.001\text{ M KCN}$ , plotted according to the equation for a first order reaction.

The  $\text{CO}_2/\text{O}_2$  quotient observed is characteristic of this type of substrate, for in the early stages of germination of *Ricinus* in which fat is being changed to carbohydrate, Murlin (1933–34) has reported respiratory quotients of from 0.30 to 0.58, and, in the spontaneous oxidation of linolenic acid at  $100^{\circ}$ , 9 atoms of oxygen



are taken up and 1 molecule of carbon dioxide is produced per mole, which corresponds to a quotient of 0.22 (Coffey, 1921). The successful substitution of linseed and castor oils for *Lupinus* oil in the system also supports the view that the substrate is the reserve oil of the seeds.

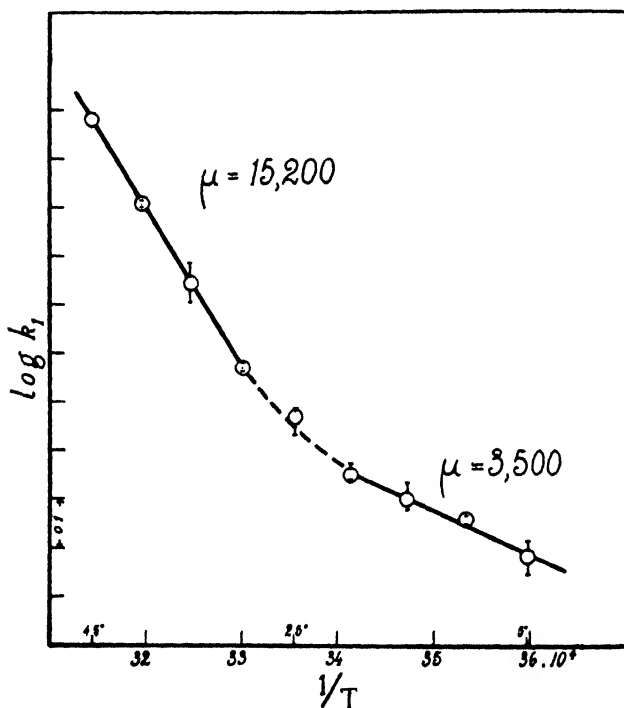


FIG. 10. Velocity constants for oxygen consumption of standard reaction mixtures with 0.001 M KCN plotted according to the Arrhenius equation. The points represent the mean of four experiments at each temperature and the maximum deviation.

While the net result of the process seems to be oxidation of a fat, it is impossible to say what the chemical reaction is.

It is clear that the enzyme component is a mixture of two or more enzymes from the following evidence: (1) cyanide may inhibit, accelerate, or have no effect; (2) the break in the heat in-activation curve.

The results suggest that a number of types of catalytic activity are involved: (1) adsorption: inhibition by urethane; (2) dehydrogenation: anaerobic carbon dioxide production; (3) oxygen activation: inhibition by carbon monoxide and cyanide; qualitative tests show the presence of indophenol and polyphenol oxidases; (4)

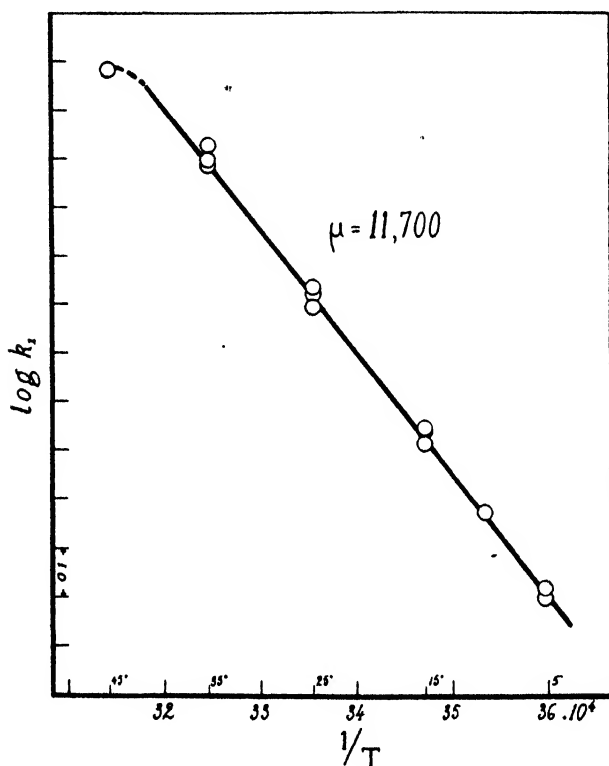


FIG. 11. Temperature characteristic for oxygen consumption in the presence of  $0.001 \text{ M KCN}$  when oil is added to the system.

cyanide-stable iron catalysis of the hemin type: at  $15^\circ$  and  $45^\circ$ , potassium cyanide does not inhibit; (5) phosphate: whether this acts in an unknown fashion as a promoter in the sense of Lyon (1927) or by the formation of active intermediate compounds as in fermentation is uncertain.

Let us now consider the behavior of the velocity constants for

oxygen consumption as a function of temperature. For the system without added oil (Fig. 10), there is a shift from a low value of the temperature coefficient in the low temperature range to a high value in the high range. This implies that the observed reaction is a composite one made up of two or more concurrent reactions differently influenced by temperature (Hinshelwood, 1929). The existence of such concurrent reactions is indicated independently by the complexity of the enzyme component as outlined above. When the system is saturated with the substrate, this shift in the temperature coefficient disappears and another value of  $\mu$  holds over the greater part of the temperature range. Although a vital process is not involved here, this behavior may give some evidence as to the nature of temperature characteristics (Crozier, 1935).

With regard to the relation between the powder-water system and the respiration of the germinating seedlings, the observation that the initial respiratory quotient of the seedlings is unity (Craig, 1936) eliminates fat oxidation from consideration as the first source of energy in germination. On the other hand, the value of  $\mu = 11,700$  calories for oxygen consumption appears with the seedlings above the critical temperature (Tang, 1930-31) and with the powder-water-oil system (Fig. 11).

These results are of further interest. Stiles and Leach (1933) have shown for *Lupinus* and other seedlings that the respiratory quotient during germination may fall considerably below 1. Also, Tang (1931-32) has found for *Lupinus albus* that the respiratory quotient of the germinating seedlings is directly dependent upon temperature. The most probable explanation of these fluctuations is that the respiratory quotient is determined to a large extent by the ratio of utilization of fat to carbohydrate.

The catalytic effect of iron compounds on the oxidation of fats and fatty acids has been mentioned above. The work of Kuhn and Meyer (1929) has shown in the case of hemin that the catalysis is insensitive to cyanide. But a considerable part of cellular respiration also is cyanide-stable, as has been demonstrated for various mammalian tissues (Dixon and Elliot, 1929), for *Lathyrus odoratus* (Genevois, 1927), and for *Chlorella* (Emerson, 1926). Accordingly, Kuhn and Meyer advance the theory that utilization

of fat by the organism may account for respiration in the presence of cyanide.

In each of these cases, the discovery of a fat oxidation system in *Lupinus* is significant.

#### SUMMARY

Pulverized seeds of *Lupinus albus*, when shaken with water in the Warburg apparatus, consume oxygen and produce carbon dioxide in a ratio of  $\text{CO}_2/\text{O}_2$  of 0.3 or less. There is a small amount of anaerobic carbon dioxide production. The gas exchange arises from the action of a mixture of enzymes upon the oil reserves of the seed. The enzyme component has been isolated in stable form.

Oxygen consumption by the system is inhibited by carbon monoxide, urethane, and under some conditions by cyanide; under others, a 40 per cent increase with cyanide was observed. Oxygen consumption is also increased in phosphate buffer. Carbon dioxide production is accelerated or increased by urethane.

Temperature coefficients for oxygen consumption have been determined. The values of  $\mu$  in the Arrhenius equation are 11,700 between 5–35° when the system is saturated with oil, and 15,200 above 30° and 3500 below 20° in the absence of added oil.

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# THE TRANSFER OF INORGANIC PHOSPHORUS ACROSS THE RED BLOOD CELL MEMBRANE\*

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The experiments recorded in this paper fall into three main sections. The first deals with the distribution of inorganic phosphorus between cells and serum. No figures were found in the literature which were entirely acceptable but the impression is gained that the concentration of inorganic phosphorus is approximately the same in cells and serum (1-15). All the reported results are open to criticism for various reasons, of which the most frequent were delay in analysis, the use of blood other than human, addition of anticoagulants, failure to prevent loss of  $\text{CO}_2$ , use of methods which do not exclude easily hydrolyzable organic phosphorus, and failure to determine cell volume and water concentration so that the concentration per unit of solvent could be calculated for cells and serum.

The second section deals with the permeability of the red cell membrane to inorganic phosphorus at  $3^\circ$ ,  $23^\circ$ , and  $37.5^\circ$ , and the third section with the influence of phosphate concentration and glucose metabolism on the distribution of inorganic phosphorus *in vitro*.

## *Analytical Methods*

Venous blood was withdrawn under oil from fasting subjects, in most cases with stasis, and transferred anaerobically to a mercury defibrinating bulb. After defibrination by shaking for 10 minutes, a portion was removed under oil and centrifuged to separate the serum.

\* This article represents work done in fulfilment of the thesis requirement for the degree of Doctor of Medicine at Yale University School of Medicine.

*Cell Volume*—Daland hematocrit tubes were filled with blood and centrifuged for 1 to 1½ hours at 1500 R.P.M. In the first five analyses duplicates were made, and repeated if the first two did not agree within 1 volume per cent; and in all the others, six to ten determinations were made simultaneously and the average used, bringing the error deviation well within 1 per cent.

TABLE I

*Comparison of Methods and Recovery of Added Phosphate; Fiske and Subbarow Method*

Specimen	Inorganic P	
	Macromethod	Micromethod
	mg. per 100 cc.	mg. per 100 cc.
Standard solution (used for comparison)	0.600	0.594
Whole blood	2.80	2.87
Equal volumes whole blood and phosphate solution to make 3.40 mg. per 100 cc.	3.59	3.56

*Accuracy of Micromethod and Recovery of Added Phosphate*

Specimen	Colorimetric reading	Inorganic P
	mm.	mg. per 100 cc.
Whole blood	13.1	12.82
	13.2	12.72
	13.2	12.72
		12.75 Average
Equal volumes whole blood and phosphate solution to make 10.38 mg. per 100 cc.	16.0	10.50
	15.8	10.63
	15.8	10.63
		10.57 Average

*Water Content*—1 or 2 cc. of whole blood or serum were measured into a small bottle and weighed. This was dried at 100° to constant weight, which required 3 or 4 days. At first these determinations were carried out in duplicate, but the agreement was always so close that single analyses only were made in later experiments.

*Inorganic Phosphorus*—The method of Fiske and Subbarow (16), reduced to micro proportions, was found to give satisfactory results. 0.2 cc. of blood or serum is precipitated directly with 1.2

cc. of 10 per cent trichloroacetic acid and centrifuged. 1 cc. of supernatant fluid is diluted with 0.7 cc. of water. 0.2 cc. of 2.5 per cent ammonium molybdate in 3 N sulfuric acid is added and the color is developed with 0.7 cc. of aminonaphthosulfonic acid reducing solution. As described in the original method, molybdate in 5 N sulfuric acid is used in the standard.

To test the accuracy of the methods, several recovery experiments were run. Typical sets of results are given in Table I. Satisfactory agreement was obtained when the micromethod was checked against the original Fiske and Subbarow macromethod.

In all the results recorded in this paper, analyses of inorganic P were made in duplicate or triplicate. The average deviations between the analyses on the same fluid were rarely greater and usually much less than 3 per cent. Whole blood and serum inorganic P were measured directly; cell inorganic P was calculated with the aid of these figures and the cell volume.

The concentration of water in the cells was calculated in a similar manner, with the cell volume and the concentration of water in whole blood and serum. Inorganic P was then expressed in mg. per 100 cc. of water in cells or serum.

*Glucose*—The micro form of the Benedict method (17) was applied to Somogyi filtrates (18), Folin-Wu sugar tubes as modified by Rothberg and Evans being employed (19).

### *I. Distribution of Inorganic Phosphorus between Cells and Serum of Human Blood*

There are twenty-eight subjects in this series, eight females and twenty males. All but one were patients on the adult medical or surgical wards of the New Haven Hospital.

The inorganic P of whole blood ranged from 2.52 to 11.58 mg. per 100 cc., only six being over 4 mg. In twenty-nine of the thirty-three determinations, there was less inorganic P per 100 cc. of water in the cells than in the serum. This difference varies from 0.42 to 9.81 mg., the greater differences occurring usually in the bloods containing the higher concentrations of inorganic P. The fractional difference of the inorganic phosphate—i.e. serum phosphate minus cell phosphate divided by serum phosphate—varies from 11 to 62 per cent and is not correlated with concentrations of phosphate in either cells or serum. The direction and



nature of the discrepancy of distribution is evident from Fig. 1. It will be seen that the great majority of points lie below the 45° line which represents equality of distribution.

In four of the thirty-three determinations, the concentration of inorganic phosphorus per unit of water was found to be greater in

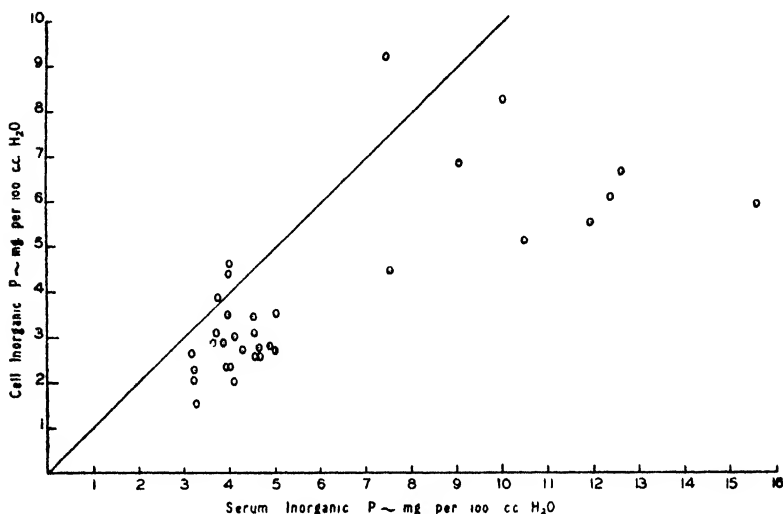


FIG. 1. Distribution of inorganic P between cells and serum

TABLE II

*Changes in Inorganic P of Whole Blood in 1 Hour at 25°*

Sample No.	P, mg. per 100 cc.	
	Immediate	Delayed
1	5.81	5.94
2	9.56	9.48
3	11.55	11.58

the cells than in the serum. Why these four cases differ from the others remains unexplained.

The possibility that these results might be due to inadequacies of technique obviously had to be considered. By employing anaerobic technique the changes in inorganic P due to loss of CO<sub>2</sub>, to which Lawaczek (20) has called attention, were avoided. In

order to minimize the increase in inorganic P which is known to occur in the cells at room temperature, the analyses were completed as rapidly as possible—within 45 minutes after the blood had been drawn. Three experiments were performed which showed that there is no significant increase in the inorganic P of anaerobic defibrinated blood in 45 to 60 minutes at 23°. Blood was drawn in the usual manner; one part was defibrinated before analysis, the other part was precipitated immediately in trichloroacetic acid and analyzed within 20 minutes. The concentration of inorganic P was about the same in both samples (see Table II).

Rapid analysis was also necessary in order to minimize the rise that is known to occur in the trichloroacetic acid medium. In accordance with the work of Sahyun (21) on rabbit blood, we

TABLE III  
*Changes in Concentration of Inorganic P in Trichloroacetic Acid Filtrate at 23°*

Sample	Time of standing <i>hrs.</i>	Inorganic P, mg. per 100 cc.		
		Before	After	Difference
1. Blood.....	7	4.50	6.17	+1.67
2. ".....	8.5	5.95	6.47	+0.52
3. ".....	9	9.48	10.81	+1.33
Serum.....	9	11.70	11.58	-0.12

observed an increase of as much as 1.67 mg. of inorganic P per 100 cc. of whole blood in 7 hours at 23°, but the concentration in the acid precipitate of serum remained constant (Table III). We do not believe, therefore, that the rise in the inorganic P of blood due to analysis in an acid medium is a matter of significance, since the procedure is completed so quickly.

Two rather remote but possible sources of error remain—instantaneous changes in the concentration of inorganic P either at the time of drawing the blood or on contact with the acid precipitant. With the limitations of these factors, which we have not been able to avoid, our results do not confirm the findings of Buell (22) who, using such special precautions as chilling of the blood and rapid analysis, found no inorganic P in the red cells of the dog. In human red cells she found as little as 0.5 mg. per 100 cc. and

believed that even this small amount was due to the breakdown of organic P after drawing the blood.

The values reported in this paper do not differentiate between red and white blood cells because of the numerical insignificance of the latter which is not counterbalanced by their relatively high concentration of inorganic P, about 20 mg. per 100 cc. according to Kay (23).

The discovery that inorganic P is in most cases less concentrated in the cells than in the serum of blood of fasting humans cannot be attributed to the Donnan effect because of the magnitude of the

TABLE IV  
*Distribution of Inorganic P in Blood after Glucose and Insulin*

	Specimen	Glucose  mg. per 100 cc. blood	Inorganic P, mg. per 100 cc.		
			Blood	H <sub>2</sub> O	
				Cells	Serum
JB	1. Control	165	2.55	2.67	3.17
	25 gm. glucose, 15 units insulin injected				
	2. 5 min. later	260	2.51	3.11	2.90
	3. 30 " "	229	2.36	2.83	2.75
	4. 1 hr. " "	204	2.18	2.99	2.36
JK	5. 2 hrs. " "	155	1.87	2.66	2.12
	1. Control	105	6.92	9.17	7.46
	25 gm. glucose injected				
	2. 1 hr. later	148	5.87	6.78	6.61
	3. 2 hrs. " "	81	5.44	4.18	6.62

difference and the fact that it tends to increase with concentration. So far as can be ascertained from the literature, all inorganic P is diffusible, none being bound in a more complex form, as is the case with calcium. But since the distribution between cells and serum is unequal, it is not likely that the red cell membrane is freely permeable to the electrolyte. If it is at all permeable, the distribution of inorganic P in the blood at any time would be the resultant of at least three factors: the interchange between serum and tissues, the constantly shifting equilibrium between organic and inorganic P within the blood cells, and the rate of diffusion across the membrane. This distribution would not be expected, therefore, to

remain always the same, but rather to reflect the recent history of the blood which is taking part in a complex dynamic process.

An indication that this theory holds some truth was obtained from the two following experiments. The distribution of inorganic P between cells and serum and the concentration of glucose in the blood of two patients were determined after the usual overnight fasting period. After 25 gm. of glucose and 15 units of insulin were injected intravenously, specimens of blood were withdrawn at intervals and analyzed for glucose and for the distribution of inorganic P. The results, recorded in Table IV, demonstrate that the concentration within the cells does not remain static when the concentration is decreasing in the serum and that the two systems are probably not wholly independent. Furthermore, it is not always true that there is more inorganic P per unit of water in the serum than in the cells; under certain conditions the relationship may be reversed.

## II. *Permeability of Red Blood Cell Membrane to Inorganic Phosphorus*

Very little information on this subject is available in the literature. The general consensus of opinion in the earlier papers (24-27) indicates that the red cell membranes of human and rabbit blood are only slightly permeable to inorganic P at 3° and that this permeability increases somewhat with a rise in temperature. Such factors as the change in rate of diffusion with time and concentration and the effect of previous manipulation of the cells were not adequately considered.

Lawaczek (20) in 1924 reported that in human blood *in vitro* a drop of pH caused a rise of inorganic P in the cells, while the converse occurred when the pH rose. This process was reversible and was accelerated by increased temperature. The addition of inorganic P favored the synthesis of organic P, while the addition of glucose had no effect. In whole blood at 37.5° the changes in the cells were reflected in the serum and not only inorganic but also some form of organic P was thought to participate in this diffusion of phosphorus between cells and serum. This conclusion was proved invalid several years later by Kay and Byrom (28) who showed that, although organic ester P was dialyzable, the selective permeability of the red blood cell membrane served

to keep it practically within the cell. This impermeability was not altered by changes in temperature or pH but, after autolysis

TABLE V  
*Impermeability of the Blood Cell to Inorganic P at 3°*

Experiment No.	Sample	Duration of experiment	Cell volume	Inorganic P, mg. per 100 cc.		
				Blood	H <sub>2</sub> O	
					Cells	Serum
Impermeability to passage of P from serum to cells						
1	(a) Initial	hrs.	per cent			
	(b) +NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub>	6	45.2	2.83	2.89	3.69
	(c) Control		43.4	13.93	2.18	25.13
2	(a) Initial		2.45	1.50	3.85	
	(b) +Na <sub>2</sub> HPO <sub>4</sub> and 1% glucose	8	44.3	3.17	3.53	3.95
			42.8	6.97	1.01	12.14
	(c) Control		2.53	1.31	4.07	
Impermeability to passage of P from cells to serum						
3	(a) Initial		32.0	8.35	8.20	10.04
	(b) +1.2% NaCl	3.75	20.2	7.33	6.66	8.73
	(c) Control		30.0	8.08	6.53	10.12
	Calculated if membrane is <i>not</i> permeable.....				12.1	8.96
	" " " " permeable.....				6.53	9.66
4	(a) Initial		34.5	3.55	3.08	4.56
	(b) +1.2% NaCl	3	22.1	4.56	10.20	4.49
	(c) Control		33.4	4.75	5.46	5.50
	Calculated if membrane is <i>not</i> permeable.....				10.40	4.67
	" " " " permeable.....				5.46	5.50
5	(a) Initial		27.3	7.50	6.82	9.08
	(b) +14% sucrose	2.5	14.9	5.87	7.81	7.37
	(c) Control		27.8	7.39	5.74	9.29
	Calculated if membrane is <i>not</i> permeable.....				13.4	7.47
	" " " " permeable.....				5.74	8.24

of the ester P at room temperature, the inorganic P diffused into the plasma.

In the present experiments the permeability of cells other than those of the blood has not been examined, but it is of interest to

mention briefly the results of three studies. Irving and Bastedo (29) and Eggleton (30) demonstrated a great disparity between the inorganic P of blood and stimulated muscle cells and found no evidence for an exchange of phosphate between them. The more recent investigations of Pollack, Bollman, *et al.* (31) led to similar conclusions, although the nature of their experiments was quite different.

*Permeability of Red Blood Cell Membrane to Inorganic P at 3°—* The distribution of inorganic P between cells and serum was determined according to the routine described in Section I. In most of the experiments, the specimen of blood was then divided into two parts, one of these to be used as a control. Nothing was added to this sample, but it was kept under the same temperature conditions as the second sample. In the first six cases dry  $\text{NH}_4\text{H}_2\text{PO}_4$  or  $\text{Na}_2\text{HPO}_4$  and glucose were dissolved in whole blood in amounts equivalent to 5.6 to 11.1 mg. of P per 100 cc. The blood was then immediately put into the refrigerator (2–3°), kept there, with occasional shaking, for 3 to 9 hours, and then analyzed again for the distribution of inorganic P between cells and serum. The results of two representative examples of these six experiments are recorded in Experiments 1 and 2 of Table V.

Although the inorganic P was as high as 25 mg. per 100 cc. in the serum, there was a definite but slight decrease in the cells in five of the six cases. There was certainly no tendency for the concentrations of inorganic P to become equal on both sides of the red cell membrane.

In the next five experiments, of which three are recorded in Table V (Experiments 3, 4, and 5), the same procedure was used except that dry sodium chloride or, in one case, sucrose was added to Sample *b* in such concentration that a definite diminution in cell volume, due to the removal of water, could be expected. In order to avoid hemolysis, the powder was dissolved in the serum which was then mixed with the cells from which it had been temporarily separated. Specimens of blood containing high initial concentrations of inorganic P were used for these experiments in order to obtain changes of sufficient magnitude to be significant.

The actual data of three of the five experiments are recorded in Table V (Experiments 3, 4, and 5) with, for comparison, theoretical

values for the phosphate of cells and serum, calculated on the basis of two alternative hypotheses: (a) that the membrane is not permeable, (b) that it is completely permeable, to inorganic P. The volume of cells and the concentration of inorganic P in the control samples are used as a basis for these calculations by means of the formulæ given below, in which the following symbols are used.

$$V_c - V_a = V$$

$V_c$  = volume of cells in 100 cc. control blood

$V_a$  = " " " " 100 " blood with added NaCl or sucrose

$V$  = cc. of water passing from cells to serum in 100 cc. blood with added NaCl or sucrose

$C_c$  = mg. of inorganic P in 100 cc. cell water (control)

$C_s$  = " " " " " 100 " serum " "

$H_c$  = cc. " water in 100 cc. cells

$H_s$  = " " " " 100 " serum

(a) If the membrane is not permeable to inorganic phosphorus

$$(1) \frac{V_c H_c C_s}{V_s H_c - 100V} = \text{mg. inorganic P in 100 cc. cell water}$$

$$(2) \frac{C_s H_s (100 - V_c)}{H_s (100 - V_c) + 100V} = \text{mg. inorganic P in 100 cc. serum water}$$

(b) If the membrane is freely permeable to inorganic phosphorus, (1) the concentration within the cell would remain unchanged;

$$(2) \frac{C_s H_s (100 - V_c) + 100 C_c V}{H_s (100 - V_c) + 100V} = \text{mg. inorganic P in 100 cc. serum water}$$

In all cases the concentration of inorganic P determined by analysis in the serum of Sample *b* is lower than in Sample *a* and agrees better with the theoretical value calculated on the assumption that the membrane is *not* permeable to inorganic phosphorus. In one case, Experiment 4, a considerable unexplained discrepancy is noted between the phosphate of the original serum and that of the control. This does not affect the interpretation, however, since Sample *b* is compared in all cases with the control.

Equally clear cut conclusions cannot be drawn from the findings in the cells. In those cases in which the concentration of inorganic phosphorus in the cells of the control can be used for comparison

as, for example, in Experiment 4, the concentration in the cells of Sample *b* agrees well with the theoretical values, calculated on the assumption that the cell membrane is not permeable to phosphate. But in Experiments 3 and 5, where the concentration in the whole blood of the control sample differs from that in Sample *b*, it is unnecessarily complicated to attempt to analyze the values for the cells. It is perfectly clear from the changes in the serum in all five experiments and in the cells in three that inorganic phosphorus does not leave the cells at 3°, even when the concentration increases to twice its original value. This conclusion is confirmed by calculating for each experiment the mg. of inorganic P which crossed the cell membrane in 100 cc. of blood. In the specimens to which hypertonic salt was added (Sample *b*) P left the cell in three cases

TABLE VI  
*Impermeability of Red Cell Membrane to Inorganic P at 23°*

Subject TH Specimen No.	Duration of experi- ment	Cell volume	Inorganic P, mg. per 100 cc.		
			Blood	H <sub>2</sub> O	
				Cells	Serum
	<i>hrs.</i>	<i>per cent</i>			
1. Anaerobic, immediate analysis.		29.9	3.64	2.76	4.67
2. Control, aerobic.....	3	29.6	3.32	1.84	4.47
3. Air bubbled through.....	3	28.7	3.12	0.51	4.54
4. CO <sub>2</sub> " ".....	3	30.5	4.52	6.79	4.63

and entered it in two. The average change was 0.36 mg. In the control specimens, inorganic P left the cells in all but one case, the average change being 0.28 mg. Since the average concentration of inorganic P was 6.3 mg. per 100 cc. of whole blood, these changes are close enough to the error of the method to be considered unimportant and are not recorded in Table V.

When the values for the nine control specimens in this series at refrigerator temperature are compared with the initial analyses, it is found that in every instance (except Experiment 4) there is a decrease of inorganic phosphorus of the whole blood, due to a fall, not in the serum, but in the cells. This is caused by the rise in pH following the loss of CO<sub>2</sub> on exposure to air, which, at 3°, is not counterbalanced by a drop in pH due to glycolysis.



TABLE VII  
Permeability of Red Cell Membrane to Inorganic P at 37.5°

	Specimen	Duration of experiment	Cell volume, per cent H <sub>2</sub> O, per cent*	Inorganic P, mg. per 100 cc.			
				Blood	H <sub>2</sub> O		Ex-change†
		hrs.			Cells	Serum	
RB	1. Anaerobic, immediate analysis		31.3 75.6 C. 94.3 S.	9.48	6.08	12.40	
	2. No addition	7.5	32.7 74.1 C. 93.8 S.	14.69	15.94	17.17	+2.79
	3. Blood + 1.2% NaCl	7.5	22.8 64.0 C. 94.2 S.	15.18	18.64	17.15	+4.43
	Calculated if membrane is permeable.....				15.94	17.00	
	" " " " not permeable.....				27.0	14.95	
NR	1. Anaerobic, immediate analysis		44.8 72.7 C. 95.0 S.	3.30	2.58	4.58	
	2. Control (no additions), 37.5°	3		3.34	3.63	4.15	-0.35
		6		6.11	7.70	6.85	+0.95
		9		13.34	15.70	15.50	+5.07
		12	49.8 75.7 C. 94.2 S.	20.26	22.00	25.4	+9.53
	3. Blood + Na <sub>2</sub> HPO <sub>4</sub> , 37.5°	3		12.91	10.75	18.2	-4.22
		6		17.50	17.80	22.6	-2.31
		9		26.45	27.9	33.7	+2.58
		12	50.6 76.9 C. 93.4 S.	32.3	34.1	41.3	+5.57
	23°	3		14.31	4.17	24.6	-0.51
		6		13.66	5.06	22.8	-1.46
		9		12.93	4.39	21.9	-2.03
		12	44.5 73.3 C. 94.5 S.	13.21	9.06	19.5	-3.25

\* The figures followed by C. indicate cc. of water per 100 cc. of cells; by S., cc. of water per 100 cc. of serum.

† Mg. of inorganic P per 100 cc. of blood transferred across the cell membrane. When the exchange has been from cells to serum, the figure is preceded by a plus sign; when from serum to cells, by a minus sign.

*Conclusion*—At refrigerator temperature, inorganic phosphorus does not enter or leave the red blood cell to any appreciable extent in periods as long as 9 hours. The concentration of inorganic phosphorus in whole blood falls, owing to a decrease in the cells under these conditions of low temperature and exposure to air.

*Permeability of Red Blood Cell Membrane to Inorganic P at 23° and 37.5°*—In one experiment the pH was changed by varying the CO<sub>2</sub> content, in order to ascertain whether fluctuations in inorganic P in the cells were reflected in the serum (Table VI). The blood was kept at room temperature, 23°, and, as at 3°, it was demonstrated that no appreciable amounts of inorganic P crossed the membranes in 3 hours, although the concentrations in cells and serum were widely different. The phosphate in the serum remained practically constant in each case, while it underwent changes of considerable magnitude in the cells.

Since no appreciable increase in the permeability of the cell membrane was produced by a rise of 20° in temperature (from 3–23°), it seemed doubtful at this point that inorganic P could cross the membrane under any ordinary conditions of concentration and temperature. The following experiments, however, indicated that at 37.5° definite evidence of the transfer of phosphate ion could be demonstrated when sufficient time was allowed. Two of the five experiments in this group are given in Table VII. These were selected for Table VII because the data are more complete as well as being representative of the results of the three unrecorded experiments.

As an indicator of the permeability of the membrane, the change in concentration in the serum is more reliable than is the change in the cells. This has already been discussed in reference to Tables V and VI. The inorganic P increases in the cells as the blood is permitted to stand in the incubator, and if the concentration in the serum rises at the same time, it can safely be concluded that diffusion has taken place across the membrane, since it is known that no rise will occur in serum at 37.5° after it has been separated from the cells.

The last column in Table VII gives the amounts of P which passed out of the cells in 100 cc. of blood, calculated according to the formula given above. When the transfer occurred in the opposite direction—from the serum to the cells—the figure is

preceded by a minus sign. The comparison is always made with the original blood or, if phosphate has been added, with the first analysis after the addition. The figures in this column indicate a definite transfer of inorganic P in both directions across the membrane at 37.5°.

This unmistakable ability of the phosphate ion to penetrate the red cell membrane at 37.5° might have been due to a loss of viability of the cells, with a consequent loss of selective permeability, or it might simply have been due to an increased rate of diffusion at the higher temperature. The increase was, however, too sharp between 23–37.5°, no evidence of permeability having been obtained thus far at or below 23°, so a third possibility was thereupon considered: that the passage of inorganic P across the membrane cannot be entirely explained by the simple laws of diffusion, but that in some way the metabolic activity which utilizes the diffusing substance exerts an important influence on the rate and direction of transfer. Obviously, the metabolic activity of the cell is greater at 37.5° than at 3° or 23°. And it might be this factor, rather than the temperature *per se*, which would determine the transfer of inorganic P. Experimental evidence for this theory is presented in the remainder of this paper. In order to clarify this issue, it was first necessary to compare the rates of diffusion at 23° and at 37.5° over relatively long periods.

In Table VII are recorded the changes in the distribution of inorganic P in the same blood (NR) at 23° and at 37.5° at 3 hour intervals during a period of 12 hours. The procedure was similar to that described for the earlier experiments. It is seen that, after the first 3 hours, the concentration of inorganic P increases rapidly in the cells at 37.5° and that this increase is reflected in the serum. During the first 3 hours at 37.5° inorganic P passed from the serum into the cells, more in the sample to which phosphate had been added than in the control. There is fairly good parallelism after the first 3 hours between the curves for the concentration in the serum of the two samples which were kept in the incubator, in spite of the fact that the sample to which  $\text{Na}_2\text{HPO}_4$  had been added contained more inorganic P and showed a greater difference between cells and serum than did the control. The increments in the serum are for the control and the phosphated blood, respectively: 3 to 6 hours, 1.30 and 2.09 mg.; 6 to 9 hours, 4.12 and 4.89

mg.; 9 to 12 hours, 4.46 and 2.99 mg.; total for 9 hours, 9.88 and 9.97 mg. If any conclusion can be drawn from the single experiment, it may be said that the direction of transfer of inorganic P across the blood cell membrane is not greatly influenced by either the actual concentration or the difference between cells and serum, but the rate of transfer may be affected.

In the sample kept at 23° to which inorganic P was added, diffusion proceeded from the serum into the cells, in the reverse direction from the other two samples, and at a much slower rate than at the higher temperature. That this difference is not due to a loss of viability of the cells at 37.5° is shown by the fact that even at the end of 12 hours there is still a difference in concentration between cells and serum. If the membrane had lost its selective permeability, it would be expected that the concentrations on the two sides would have become rapidly equalized. On the other hand, the increase in the rate of transfer across the membrane at 37.5° is entirely too large to be satisfactorily attributed wholly to an increase in the activity of the phosphate ion with the rise of temperature. It can be seen from the experimental data that inorganic P diffuses from a lower to a higher concentration when it is being rapidly liberated from the organic form in the cells. On the other hand, when the reaction is proceeding slowly in the opposite direction—inorganic P being transformed to organic P—as it is at 23°, the ion diffuses into the cell at a correspondingly slow rate.

*Conclusions*—Transfer of inorganic P across the blood cell membrane can readily be demonstrated at 37.5°. The same process occurs at 23° but so slowly that it is only appreciable after long periods. It is strongly suggested that the marked increase in the rate of transfer at the higher temperature is attributable, not to a loss of viability of the cells or an increase in permeability of the membrane nor to an increase in concentration or activity of the phosphate ion, but to a response to an increased metabolic activity which involves the phosphate ion.

### *III. Relation of Carbohydrate Metabolism in Red Blood Cell to Transfer of Inorganic P across Membrane*

The participation of the red blood cell in carbohydrate metabolism is still an unsettled question (5, 7, 8, 12, 32-34), although it is known that most of the intermediary compounds present in muscle

**TABLE VIII**  
*Influence of Glycolysis on the Inorganic P of Blood at 37.5°*

	Specimen	Time	Glucose	Cell volume, per cent H <sub>2</sub> O, per cent*	Inorganic P, mg. per 100 cc.			
					Blood	H <sub>2</sub> O		Ex-change†
						Cells	Serum	
		hrs.	mg. per 100 cc. blood					
NB	1. Anaerobic, immediate analysis			33.8	2.57	1.57	3.29	
				70.5 C.				
				93.7 S.				
				34.2	6.38	7.90	7.28	+2.53
	2. Control	5		70.4 C.				
				93.4 S.				
		0	788		9.16			
		5	743	34.4	11.55	10.5	14.7	+0.35
	3. Blood + Na <sub>2</sub> HPO <sub>4</sub> + glucose			70.5 C.				
				93.2 S.				
RB	1. Anaerobic, immediate analysis			191	30.1	11.6		
					69.0 C.			
					94.5 S.			
		2	136		12.2			
	2. Control	7	61		15.3			
		12	<24		18.2			
		2	165		13.6			
		7	180		17.2			
	3. Blood + 0.2% NaF	12	167		20.9			
LA	1. Anaerobic, immediate analysis			92	44.5	2.90		
					70.9 C.			
					92.3 S.			
		2	57		3.04			
	2. Control	4	36		3.47			
		6	24		4.96			
		8	<24		10.2			
		10	<24		15.3			
	Glucose added	12	51		13.0			
		2	83		4.83			
		4	89		6.56			
		6	92		7.72			
	3. Blood + 0.2% NaF	8	87		9.03			
		10	87		9.47			
		12	77		9.73			

\* The figures followed by C. indicate cc. of water per 100 cc. of cells; by S., cc. of water per 100 cc. of serum.

† Mg. of inorganic P per 100 cc. of blood transferred across the cell membrane. When the exchange has been from cells to serum, the figure is preceded by a plus sign; when from serum to cells, by a minus sign.

TABLE VIII—*Concluded*

	Specimen	Time	Glucose	Cell volume, per cent $H_2O$ , per cent*	Inorganic P, mg. per 100 cc.			
					Blood	$H_2O$		Ex-change†
						Cells	Serum	
		hrs.	mg. per 100 cc. blood					
DN <sub>1</sub>	1. Anaerobic, immediate analysis		110	47.6 73.2 C. 94.8 S.	2.80	2.38	3.98	
	2. Control	3 "	24		3.63	3.93	4.60	+1.00
		6	<24		9.10	11.1	10.5	+2.86
		9	<24		17.1	19.0	21.4	+7.47
		12	<24	55.0 76.2 C. 94.1 S.	24.1	25.4	31.7	+11.47
	3. Blood + $Na_2HPO_4$	Immediate			13.91	2.38	26.4	
		3	28		15.2	14.0	21.1	-2.97
		6	<24		22.0	23.0	28.7	+0.13
		9	<24		31.4	35.2	39.1	+4.18
		12	<24	54.9 76.0 C. 94.1 S.	36.2	45.3	40.7	+4.13
	4. Blood + $Na_2HPO_4$ + 0.15% NaF	Immediate			13.9	2.38	26.4	
		3	66		16.8	15.9	22.5	-1.42
		6	94		19.3	17.3	26.1	+1.03
		9	85		20.8	18.5	27.8	+2.53
		12	93	38.2 67.1 C. 94.6 S.	21.4	20.1	27.8	+3.18
DN <sub>2</sub>	1. Anaerobic, immediate analysis		116	51.0 73.2 C. 93.7 S.	2.71	2.35	4.00	
	2. Blood + $Na_2HPO_4$	Immediate			18.1	2.35	37.4	
		3	<30	50.1	16.1	12.2	24.9	-5.55
		6	<30		24.1	23.9	33.2	-2.02
	161 mg. glucose per 100 cc. added	9	120		23.0	22.5	32.1	-2.87
		12	68	52.9 74.7 C. 92.6 S.	24.6	24.6	34.0	-2.39
	3. Blood + $Na_2HPO_4$ + 0.1 % NaF	Immediate			18.1	2.35	37.4	
		3	74	43.3	21.4	17.8	30.5	-1.18
		6	86		23.7	18.2	34.7	+1.02
	161 mg. glucose per 100 cc. added	9	247		24.3	18.0	36.0	+1.62
		12	244	43.9 69.5 C. 93.0 S.	25.0	20.2	36.0	+1.56

cells are also present in the blood cell. Direct experiments on living animals have shown that when carbohydrate metabolism is accelerated, the inorganic P decreases in both cells and serum, but it has not been demonstrated that these changes are analogous to those which occur in incubated blood. *In vitro* definite relationship has been found between organic acid-soluble P, inorganic P, and glucose. During glycolysis acid-soluble hexosephosphate esters are formed which are present exclusively in the cells and, at the same time, acid-soluble organic P is breaking down to liberate inorganic P, but no reducing substance. The latter process is favored by an acid medium, the former by an alkaline medium. The actual concentration of inorganic P in the blood at any time is the resultant of these two reactions. The following experiments were undertaken mainly to observe the effect of changes in the concentration of inorganic P in the blood cell during glycolysis upon the transfer of the electrolyte across the membrane and, incidentally, to see whether there is anything essentially different between the variations in glucose and inorganic P *in vivo* and *in vitro*.

*Experimental Work*--In Section I of this paper (Table IV) it was found that when carbohydrate metabolism was accelerated *in vivo* by the injection of glucose and insulin, the concentration of inorganic P decreased in the cells as well as in the serum, although there was probably an initial transitory rise in the cells immediately after the injection. These experiments were not pursued further because, as already stated, it is more instructive at present to study the metabolism of the blood cell *in vitro* to simplify the problem by removing the possibility of interchange with the tissues and interstitial fluid.

In the experiments recorded in Table VIII the concentrations of the participating substances were altered directly by the addition of glucose and  $\text{Na}_2\text{HPO}_4$  and indirectly by the addition of NaF to prevent the disappearance of glucose. The relation of glycolysis to the concentration of inorganic P in the blood was then followed at intervals, for a period of 12 hours. Two similar experiments have been omitted for the sake of brevity. In the last two experiments in Table VIII ( $\text{DN}_1$  and  $\text{DN}_2$ ) glycolysis and phosphate concentration were again modified by the addition of NaF, glucose, and  $\text{Na}_2\text{HPO}_4$  in order to ascertain the distribu-

tion of inorganic P between cells and serum at different rates of carbohydrate breakdown.

Examination of the results of these experiments confirms the findings of the experiment on NR (Table VII). The inorganic P of incubated blood increases slowly until glycolysis is complete, after which the rise becomes much more precipitate. If the initial concentration of glucose is high, as in Experiment RB, the initial phase of the inorganic P curve is prolonged. When glucose is added during the rapid liberation of inorganic P, the rise in the second phase of the curve is definitely checked (Experiments LA and DN<sub>2</sub>). Contrary to the findings in these experiments, Lawaczek (20) has stated that the addition of phosphate shifted the equilibrium between organic and inorganic P in the direction of synthesis, while glucose had no effect. The rise in inorganic P in blood to which phosphate has been added parallels quite closely the rise in the control (Experiments NR, Table VII, and DN<sub>1</sub>, Table VIII), indicating that the concentration of inorganic P has not, in itself, an effect on the equilibrium under ordinary conditions. The presence of glucose has, on the other hand, a very definite influence in shifting the equilibrium in the direction of synthesis under conditions in which glycolysis is favored.

When glycolysis was inhibited by the presence of NaF, the changes in phosphorus followed quite a different course. The increase in inorganic P was at first more rapid than in the control but the curve soon flattened out. The addition of glucose did not alter the curve at all in one case, DN<sub>2</sub>.

It is of interest to note that in all the samples containing NaF (Experiments RB, DN<sub>1</sub>, and DN<sub>2</sub>) there was an initial decrease in the concentration of the reducing substance, then a slight rise, and again a decrease. The initial drop is probably explained by the fact that the blood remained at room temperature during the preliminary analysis for some 2 hours before fluoride was added. The subsequent rise which is slight, but consistent, may indicate a liberation of reducing substance with the breakdown of inorganic P. It must be remembered in the interpretation of these figures that, at incubator temperature, fluoride inhibits glycolysis but probably does not abolish it entirely and the concentration at any time is the resultant of two opposite reactions.

The findings in these experiments are consistent with the theory



that the breakdown of ester P and the synthesis of inorganic P may proceed simultaneously under the proper conditions. During glycolysis organic P is breaking down at the same time that inorganic P is being utilized for the formation of an acid-soluble ester, and therefore the initial rise in concentration of inorganic P in the control is less than in the blood in which glycolysis has been inhibited with fluoride. After the glucose has been exhausted, the effective rise of inorganic P is rapid and continues for a longer time in the control because of the greater reserve of ester P which has been built up. When glucose is added to the control, there is again a synthesis and, although inorganic P is still being liberated, the rise in actual concentration is checked.

These changes cannot be accounted for by variations in the hydrogen ion concentration during the course of the experiment. In Experiment LA it is seen that the concentration of inorganic P rose rapidly after the completion of glycolysis (at 5 hours), which is known to be accompanied by a fall in pH. After glucose had been added at 10 hours, there was a prompt drop in inorganic P, although, with the resumption of glycolytic activity, a further drop in pH is to be expected. It would be unreasonable to attribute the first rise in inorganic P to an increased acidity in the blood, when, with a further increase, the inorganic P falls.

These experiments confirm the impressions gained from Table VII on the effect of glycolysis on the transfer of inorganic P across the cell membrane. Examination of the last column of Experiments DN<sub>1</sub> and DN<sub>2</sub> in Table VIII reveals that, in general, when the glucose concentration is falling, inorganic P passes into the cells from the serum. When glycolysis is inhibited by NaF or completed, inorganic P leaves the cell to enter the serum, even though the concentration in the serum may already exceed that in the cell. This relation of the transfer of inorganic P across the blood cell membrane, not to the concentration gradient, but to the metabolic activity involving phosphorus in the cell, takes the process out of the realm of simple diffusion. It demonstrates that the transfer of substances across the cell membrane cannot always be explained by the simple physicochemical law of diffusion from a higher to a lower concentration, even with the modification described by Donnan, but that the rate of utilization within the cell has an important influence on the process.

There is, of course, no direct evidence in these experiments that inorganic phosphate as such crosses the cell membrane. It is even possible that the phosphorus compounds here considered as inorganic phosphate actually consist of extremely labile organic compounds. The latter hypothesis could hardly apply to the serum, since added inorganic phosphate behaved quite like that already in the serum. Against the hypothesis that the cell membrane is unconditionally impermeable to inorganic phosphorus and that exchanges involve organic esters some more recent experiments may be cited. It has been demonstrated by Wakeman, Eisenman, and Peters (35) that inorganic potassium will not penetrate the red cells of well preserved or "resting" blood, even when its concentration in the serum is greatly increased. However, Hald and Laviertes (36) have shown that when whole blood is incubated for 10 to 16 hours, in addition to phosphate a small, but definite, quantity of potassium passes from cells to serum. The concentration of potassium in serum increased 50 to 80 per cent in experiments in which there was no sign of hemolysis. It would be little short of presumptuous to suggest that potassium formed non-ionized compounds with some organic substance in the blood. Nevertheless, its transfer across the cell membrane seems to be conditioned by the same factors which control transfers of phosphate. This type of transfer across living membranes from lower to higher concentrations is not unique in the blood cell but has an analogy in the process of reabsorption in the tubules of the kidney.

The viability of the cells in these experiments is indicated in almost every case by the fact that a difference is maintained between the concentrations of phosphate in cells and serum, whereas the membrane would be expected to lose its selective permeability upon the death of the cell, after which the concentrations on either side would rapidly become equalized. It may be concluded, then, that the changes which have been observed in this study to occur in blood outside the body are analogous to those which occur in the living subject.

*Conclusions*—The red blood cells participate actively in carbohydrate metabolism. The utilization of glucose by the cell involves the preliminary formation of some sort of compound with inorganic P which is again liberated during the subsequent stages

of the catabolic change, probably accompanied by the liberation of a reducing substance as well. The transfer of inorganic P across the blood cell membrane occurs in response, not to a change of concentration gradient across the membrane, as in simple diffusion, but to the rate and direction within the cell of those metabolic processes which involve the electrolyte.

#### SUMMARY

The distribution of inorganic P between cells and serum has been determined in thirty-three instances in the blood of twenty-eight adults. In twenty-nine of the thirty-three analyses, more inorganic phosphorus was present per unit of water in the serum than in the cells, the differences ranging from 0.42 to 9.81 mg. per 100 cc. of water, or 11 to 62 per cent. This inequality in the distribution of inorganic P in human blood could be ascribed neither to technical errors nor to the Donnan effect.

Inorganic P diffused across the red cell membranes not at all at 3°, and only at a very slow rate at 23°, but at 37.5° the rate of diffusion was greatly accelerated. Diffusion was tested by noting whether inorganic phosphate added to the blood would enter the cell, or whether it would be carried out of the cell with water after the addition of hypertonic NaCl or sucrose solutions. Variations in the concentration of inorganic P within the cell, produced by incubating the blood or by changing its pH, were reflected in the serum, these changes in the concentration indicating that the ion traversed the membrane.

The increase in the rate of diffusion at 37.5° was too great to be accounted for by any of the factors which ordinarily influence simple diffusion and seemed to be related to the metabolism of glucose within the red cell rather than to the temperature *per se*. When carbohydrate metabolism *in vivo* was accelerated in two cases by the injection of glucose and insulin, the concentration of inorganic P decreased in both cells and serum. Variations in carbohydrate metabolism were then induced *in vitro* at incubator temperature by the addition of glucose and NaF, and the subsequent changes in the distribution of inorganic P were observed. Inorganic P entered the cell during glycolysis, but left the cell when glycolysis was completed, even though its concentration was already higher in the serum than in the cells. The fact that a

concentration gradient was maintained across the membrane even after an incubation period of 12 hours attests to the viability of the cells under the experimental conditions and permits analogies to be drawn between the behavior of the cells *in vitro* and *in vivo*, both with regard to metabolic activity and to membrane permeability.

The simple laws of diffusion which depend on concentration gradients do not explain the transfer of inorganic P across the red blood cell membrane, even when the variables of concentration, pH, and activity of the ions are considered. The rate and direction of exchange seem to depend primarily on the rate of those metabolic processes within the cell in which the electrolyte is involved.

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# THE EFFECT OF PARENTERAL INJECTION OF AMINO ACIDS AND RELATED SUBSTANCES UPON CREATININE FORMATION AND STORAGE IN THE RAT\*

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A statistical study of the relation between the excretion of creatinine nitrogen and several body measurements was begun by the senior author (1) in Cleveland in 1925, the results of which showed that the excretion of creatinine nitrogen was not necessarily influenced by body weight of the individual. This finding led to the study of Beard and Barnes (2) of the effect of feeding proteins, amino acids, and related substances upon creatine-creatinine metabolism. When 1 gm. quantities of different amino acids were fed to young rats for 1 day, average increases above normal in muscle creatine were obtained from 12.5 per cent for alanine to 37.5 per cent for cystine. At first these results seemed improbable, since it is very difficult to understand how all amino acids can stimulate creatine formation or be transformed into creatine. For this reason several years work was done on the subject before final publication of the original results.

In our most recent study (3) of creatine formation from proteins and amino acids, normal adult rats were fed complete synthetic diets containing either 4 per cent of casein or egg albumin as the

\* A preliminary report of these studies was given before the Thirtieth annual meeting of the American Society of Biological Chemists at Washington, D. C., March, 1936.

The data are taken in part from a thesis submitted by Thomas S. Boggess, to the Graduate School of Louisiana State University, June, 1936, in partial fulfilment of the requirements for the degree of Master of Science.

sole protein for a period of 5 weeks. Refeeding these animals on 25 per cent of casein or egg albumin, or 21 per cent of glycine or glutamic acid, caused average increases in muscle creatine varying from 20 to 56 per cent above normal. Thus partial protein starvation permits somewhat larger increases in muscle creatine after refeeding proteins and amino acids than is the case when normal well fed animals are employed. It is of interest in this connection that Borsook and Jeffreys (4) observed a new formation of creatine when a complete enzymatic hydrolysate of egg albumin was added to isolated tissues of the rat. The most significant increases in creatine occurred in the case of liver tissue.

We have emphasized the point that a concentration of free amino acids above normal must be present in the tissues, such as the muscles, if increased creatine formation is to be observed. According to Luck (5), glycine is the only amino acid that diffuses freely into muscle. In the present study we have injected the pure amino acids into the animal, thus favoring their deposition above normal in the tissues. We also wished to compare creatine formation from different amino acids with the idea of finding one that could replace glycine in the treatment of the myopathies. The results obtained show that parenteral injection of 100 mg. of the different amino acids gave increases in muscle creatine comparable to those obtained by feeding 1 or 1.5 gm. by mouth.

#### EXPERIMENTAL

Two large groups of normal rats were used. There were 60 control and 155 experimental animals in the first group. They weighed between 150 and 250 gm. and had been fed since birth on a diet of one-third skim milk powder and two-thirds whole wheat flour, with NaCl and CaCO<sub>3</sub>, each as 1 per cent of the weight of the wheat. Lettuce was included three times a week. The lettuce was omitted in all experiments reported in this paper. We also wished to confirm the results obtained with these animals with those from another colony. We secured a group of young rats weighing from 60 to 120 gm. from a breeder in New York. In this group there were twenty control and forty-five experimental animals.

The pure amino acids were obtained from Hoffmann-La Roche and were dissolved in physiological saline. A dose of 100 or 200

mg. was injected intraperitoneally at 10 a.m. on a given day. At the end of 1, 2, 3, or 4 days, the animals were killed by a blow on the back of the head and samples of muscle (hind leg), liver, and stomach were prepared and analyzed for creatine (total creatinine) by the method of Rose, Helmer, and Chanutin (6). With three persons working at the same time, all tissues were removed from the animal, thoroughly minced, weighed, and placed under the acid within 10 minutes after death. The stomach was removed and its contents washed out before weighing. The histidine dihydrochloride was neutralized just before injection. Arginine was injected as the free base. Glycocyamine and creatine were dissolved in water with the aid of a few drops of 15 per cent HCl. The injection of physiological saline gave no increase above normal in muscle creatine. Most colorimetric comparisons of total creatinine in the tissue filtrates were made independently by each of us with good checks in most cases. Plenty of food and water were always available and no pathological changes occurred in any of the animals. Injections of methylguanidine, however, caused the death of eight out of seventeen animals.

#### RESULTS AND DISCUSSION

The maximum, minimum, and average values, with per cent increases for muscle, stomach, and liver creatine obtained for all animals, are given in Table I. The effect of amino acids on creatine storage in the muscles for creatine, glycocyamine, arginine, histidine, and glycine is given in Fig. 1. The average muscle creatine of the 60 larger control animals was 0.39 per cent. This agrees with the value of 0.40 per cent obtained for 118 young animals fed on the same diet in our previous work (2). The average normal stomach and liver creatine of the larger animals was much higher than that of the smaller ones (0.21 and 0.09 per cent as compared to 0.11 and 0.04 per cent, respectively). All control and experimental animals were of comparable ages and body weights and had been fed on the stock diet mentioned above for some time in order to stabilize the creatine metabolism before injection of the various substances was made.

We attach much significance to the fact that, with only a few exceptions, the *minimum concentration* of muscle creatine in all injection experiments was the same as or higher than the *maximum*





	9	200	1	0.67	0.46	0.54	38.5	0.39	0.22	0.30	42.9	0.15	0.09	0.11	22.2
Arginine.....	4	200	2	0.60	0.43	0.53	35.9	0.10	0.04	0.08	None	0.08	0.04	0.06	None
".....	10	200	1	0.50	0.36	0.41	5.1	0.18	0.08	0.11	"	0.09	0.05	0.07	"
Glycine.....	4	200	2	0.49	0.38	0.42	7.7	0.09	0.04	0.07	"	0.09	0.04	0.06	"
".....	4	200	2	0.57	0.45	0.51	30.7	0.10	0.08	0.09	"	0.06	0.05	0.05	"
Histidine.....	5	100	3	0.42	0.38	0.40	2.5	0.10	0.09	0.10	"	0.09	0.08	0.08	"
".....	20			0.42	0.26	0.34		0.17	0.07	0.11		0.06	0.03	0.04	
Controls.....				0.49	0.43	0.47	38.2	0.13	0.11	0.11	"	0.06	0.03	0.04	"
Arginine.....	5	100	2	0.44	0.41	0.42	23.5	0.13	0.09	0.11	"	0.05	0.04	0.04	"
".....	5	100	3	0.39	0.33	0.36	5.9	0.12	0.09	0.10	"	0.05	0.04	0.04	"
".....	4	100	4	0.46	0.45	0.45	32.3	0.11	0.10	0.11	"	0.06	0.03	0.04	"
Glycine.....	5	100	1	0.31	0.25	0.28	None	0.08	0.07	0.07	"	0.04	0.03	0.03	"
".....	5	100	2	0.44	0.37	0.40	17.6	0.13	0.10	0.11	"	0.05	0.04	0.04	"
Histidine.....	5	100	2	0.43	0.39	0.40	None								
Methylguanidine†.....	4	50	1	0.43	0.40	0.42	7.7								
".....	2	50	2	0.43	0.40	0.42	7.7								
".....	1	100	1	0.43	0.43	0.43	10.2								
".....	2	100	2	0.44	0.40	0.42	7.7								
Methylguanidine.....	3	50	1	0.46	0.39	0.43	10.0								
Glycine.....		100													

\* Since there was no increase in stomach and liver creatine in the creatine and glycoeyamine experiments when 100 ng. were injected for 1 day, these determinations were omitted for the 2, 3, and 4 day studies.

† Stomach and liver creatine determinations were not made in the methylguanidine experiments.

concentration of muscle creatine in the control animals. This is especially so 2 days after the injection of the amino acids.

*Creatine, Glycocyamine, and Methylguanidine*—Creatine formation from 100 mg. of creatine or glycocyamine is less than 10 per cent for the 1st day, but at the end of the 2nd day the increases (25 to 29 per cent) are about the same as those obtained with the other amino acids at the end of 1 day. These increases have

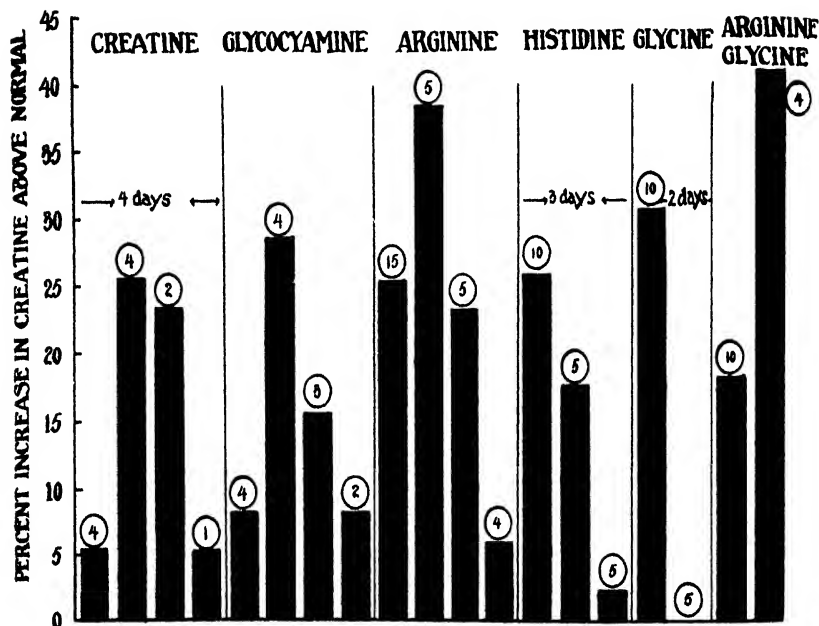


FIG. 1. Creatine storage from 1 to 4 days following the injection of amino acids and related substances. The numbers in circles represent the number of animals used in each day's study.

almost disappeared at the end of the 4th day. Wishart (7) injected guanidine sulfate into different species of animals. Increases in muscle creatine from 9 to 28 per cent above normal were obtained. In our experiments the toxicity of methylguanidine depended upon the animal. Doses from 50 to 200 mg. caused the death of eight animals. The simultaneous injection of glycine did not prevent this toxicity. In nine other animals 50 to 100 mg.

doses showed no toxicity except muscular tremors for a short time following injections. These doses of methylguanidine caused less than a 10 per cent increase in creatine formation. In three animals the 50 mg. of methylguanidine with 100 mg. of glycine prevented the increased creatine formation that is obtained from this amount of glycine alone. Further work must be carried out in regard to creatine formation with guanidine compounds before any conclusions can be drawn.

*Arginine and Histidine*—In our previous work (2) these two amino acids gave about the same increases in muscle creatine (24 per cent for histidine and 27 per cent for arginine). In the present experiments 100 mg. of each, injected for 1 day, again gave about the same increase in muscle creatine (26 per cent for histidine and 23 per cent for arginine). At the end of 2 days the arginine effect rose to 38 per cent as compared to a drop to 18 per cent for histidine. At the end of the 3rd day the arginine effect dropped to about 24 per cent, while the histidine effect dropped to about 3 per cent. Thus creatine formation from these amino acids was about the same during the first 24 hours after injection, but creatine storage was somewhat better after arginine than after histidine. These amino acids in larger doses (200 mg. for 1 or 2 days) gave increases from 30 to 40 per cent above normal.

Thompson (8) observed in many experiments with dogs, ducks, and rabbits that arginine gave rise to creatine. The injection of methyl citrate with arginine into dogs increased the creatine output over that obtained from arginine alone. In autolysis and liver perfusion experiments Takahashi and Kumon (9) observed that the methylation of arginine resulted in the formation of creatine. Tripoli and Beard (10) also suggested that creatine may be formed by the methylation of arginine. Hongo (11) observed the transformation of arginine into creatine in the gastric mucosa of dogs. Edlbacher and Röthler (12) stated that part of the ingested arginine may escape the action of liver arginase and be oxidized and converted into creatine. Thus the possibility that arginine can serve as a precursor of creatine is evident from the work reviewed above.

*Arginine Plus Glycine*—Creatine formation after the injection of 100 mg. of either of these amino acids for the first 24 hours was about the same. However, creatine storage in these cases was

markedly different. This is a point that may have some clinical significance in the future treatment of the myopathies. After the 1st day all the creatine formed from glycine has disappeared from the muscle, and even feeding 200 mg. of glycine for 1 or 2 days gives only slight increases above normal. This fact shows that 100 mg. of this amino acid injected for 24 hours is an optimum dose of glycine for creatine formation and doses above this would probably be wasted. This is probably the reason why glycine usually gives a very large increase in the creatinuria observed soon after its first ingestion by patients suffering from the various myopathies (10, 13, 14). Freiberg and West (15) and Shorr, Richardson, and Wolff (16) have shown that the ability of the patient with pseudo-hypertrophic muscular dystrophy and Graves' disease to synthesize glycine is not impaired. Since glycine increases both the creatine content of the muscles and creatine excretion in the urine of the myopathic patient, it is evident that this creatine is formed from exogenous as opposed to endogenous glycine. Other workers (17-20) also believe that the transformation of glycine to creatine is direct.

When 100 mg. each of arginine and glycine were injected together, the increase in muscle creatine 1 and 2 days later was 18 and 41 per cent, respectively. Since 100 mg. of arginine for 2 days gave a 38 per cent increase, while a similar dose of glycine for the same length of time gave no increase in muscle creatine, it is possible that there was no summation of the effect of the amino acids on creatine formation in this case. The muscles, when presented with equal quantities of these two amino acids, form creatine preferably from arginine rather than from glycine. The results on creatine formation and storage from amino acids shown in Fig. 1 would seem to indicate that arginine exerts a somewhat greater influence on creatine metabolism as shown by (a) greater creatine formation the 1st day than was observed with creatine itself, (b) a larger creatine formation the 2nd day when the glycine effect is nil, and (c) greater storage the 3rd day than is caused by any other substance. The results obtained with other substances, however, show that arginine does not possess a unique significance in creatine metabolism.

*Alanine, Valine, and Serine*—In our previous work alanine and valine fed in 0.50 to 1.0 gm. quantities for 1 day by mouth to young

rats caused an average increase of 12.5 and 34.3 per cent, respectively, in muscle creatine. In the present study 100 mg. quantities of alanine, valine, and serine each gave identical increases (23.1 per cent) in muscle creatine. These increases are similar to those obtained for 100 mg. of arginine, histidine, and glycine for 1 day. It is of interest in this connection that Decherd, Herrmann, and Davis (21) and MacKay and Barnes (22) found that alanine alone of several amino acids definitely raised the creatine content of heart muscle.

The increased creatine formation observed in the present studies was probably not the result of a "stimulation" process, either of the endogenous metabolism or of creatine formation. Borsook and Keighley (23) observed that neither the endogenous nor continuing metabolism was stimulated by the amino acids. We have also shown that refeeding proteins and amino acids to partially protein-starved rats did not cause an increase in the total nitrogen in the muscles (3) and that amino acid therapy had little effect upon the distribution of nitrogen in the urine in three cases of myopathies in the form of the total, urea, ammonia, uric acid, and undetermined nitrogen (14). In this connection Milhorat and Wolff (24) have stated, "The effect of glycine on the metabolism of creatine in muscle disease, as shown by the creatinuria and creatine tolerance, is out of all proportion to the effect of comparable amounts of nitrogen from sources other than glycine or creatine. It is postulated that glycine acts as a precursor of creatine."

When the amino acids cause an increase of creatine up to 50 per cent above normal in the muscles, and glycine and glutamic acid may cause increases up to 1000 per cent in creatine excretion in some cases of the myopathies, it is clearly seen that creatine metabolism in the organism is unstable and may be easily affected by many factors (proteins, amino acids, nuclear material, choline, various hormones, muscular diseases, fever, etc.) in the body. Therefore it is our belief that creatine arises in the body from its exogenous precursors, chiefly proteins and amino acids.

We are well aware of the fact that the exogenous origin of creatine from amino acids is not accepted by many students of creatine metabolism at the present time. However, it should be remembered in this connection that most of the negative results

have been obtained in experiments in which small doses of amino acids were fed or injected and the excretion of creatine and creatinine in the urine was used as an index of creatine formation in the body. In these cases creatine may have been formed and stored in the muscles and not excreted into the urine. Also at the present time some workers in this field are beginning to deny any relationship between creatine and creatinine in metabolism. Thus it hardly seems necessary to point out the fact that creatine formation in the muscles is an entirely different physiological process from creatine and creatinine excretion in the urine.

It is well known, especially from clinical studies in the myopathies, that amino acid ingestion may, or may not, increase the excretion of creatine and creatinine in the urine. Thus the results of the studies of Bodansky (25) and Shapiro and Zwarenstein (26), which were designed to check some of the results previously reported by Beard and Barnes (2), are of no significance upon creatine formation in the muscles, since these investigators studied, as is usually the case, only the effect of amino acids on the creatine and creatinine excretion in the urine. A review of the literature fails to reveal a single study, with our published technique, which either confirms or denies our findings with reference to creatine formation from amino acids in the muscles. We feel, therefore, that our results should be recognized until sufficient evidence to the contrary is published.

#### SUMMARY

Amino acids and related substances were injected into normal rats, and the effect of this treatment upon the creatine content of the muscle, stomach, and liver was determined from 1 to 4 days after injection. 80 control and 200 experimental animals were used. The results were as follows:

1. Parenteral injection of 100 mg. of creatine, glycocyamine, arginine, histidine, glycine, alanine, serine, or valine for 1 day gave average increases in muscle creatine in the rat varying from 6 to 32 per cent above normal. At the end of 2 days the increase for the first four substances varied from 16 to 40 per cent, most of which had about disappeared at the end of 4 days after injection.

2. The increased creatine formation from the injection of 100 mg. of glycine had disappeared at the end of 2 days.

3. Parenteral injection of 200 mg. doses of arginine and histidine for 1 or 2 days gave a 30 to 40 per cent increase in muscle creatine, while 200 mg. of glycine for 1 or 2 days caused less than 10 per cent increase above normal.

4. The injection of 100 mg. of arginine and 100 mg. of glycine together for 2 days gave a 41 per cent increase in muscle creatine. This effect was due to arginine alone.

5. 200 mg. of arginine for 1 day caused a 43 and 22 per cent increase, respectively, in stomach and liver creatine, while 100 mg. of histidine for 1 day caused a 19 per cent increase in stomach creatine. These increases disappeared at the end of 2 days.

6. Methylguanidine hydrochloride caused only a slight increase in muscle creatine in some animals and glycine did not prevent its toxicity in others.

7. Creatine formation from its precursors occurs chiefly in the muscles and maximum increases are observed 2 days after injection.

8. The exogenous origin of creatine from amino acids and related substances and creatine storage were discussed.

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**Changes in the Oxygen Content of Venous Blood As the Result of Fever Therapy with and without the Administration of Oxygen.**

BY MILDRED ADAMS AND WALTER M. BOOTHBY. *From the Section of Clinical Metabolism and Division of Medicine, The Mayo Clinic and The Mayo Foundation, Rochester, Minnesota*

Determinations were made of the oxygen content of the venous blood of subjects before fever therapy and at intervals during the treatment. The added effect of the inhalation of 50 to 80 per cent oxygen on the venous content was also determined. With body temperatures of from 38.8–41.1° a marked elevation of the oxygen content of the venous blood was observed. When the body temperature was elevated and oxygen was also administered, the percentage oxygenation of the hemoglobin frequently was found to exceed 95 per cent.

**The Composition of Leprosin.** BY R. J. ANDERSON, J. A. CROWDER, M. S. NEWMAN, AND F. H. STODOLA. *From the Department of Chemistry, Yale University, New Haven*

A substance named leprosin has been isolated as a snow-white amorphous powder from the alcohol-ether extract of *Bacillus lepræ*. Leprosin melts at about 50° and has a dextrorotation of about 4°. On saponification the substance yields a series of ordinary fatty acids from myristic acid to tetracosanic acid and a new optically active hydroxy acid of high molecular weight called leprosinic acid.

The only water-soluble constituent that could be detected was glycerol.

The unsaponifiable matter contained two optically active secondary aliphatic alcohols which were identified as *d*-eicosanol-2,  $\text{CH}_3(\text{CH}_2)_{17}\cdot\text{CHOH}\cdot\text{CH}_3$ , and *d*-octadecanol-2,  $\text{CH}_3(\text{CH}_2)_{15}\cdot\text{CHOH}\cdot\text{CH}_3$ .

**The Hydrolysis of Hair.** BY JAMES C. ANDREWS. *From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*

The observation that complete hydrolysis of human hair by means of 20 per cent hydrochloric acid requires longer boiling with large lots of hair than with smaller ones has been explained by the catalytic effect of the glass surface. This hypothesis is substantiated by comparative hydrolyses of different lots of hair under identical conditions (at 38°) in the presence of different amounts of glass wool. 50 gm. of glass wool in the presence of 500 gm. of hair reduced the time necessary to effect complete hydrolysis (negative biuret test) from about 550 days to about half that amount.

Comparison of the rate of hydrolysis of hair at 38° by means of equivalent normalities (6 N) of HCl, H<sub>2</sub>SO<sub>4</sub>, and H<sub>3</sub>PO<sub>4</sub> has led to the following results: In HCl and H<sub>3</sub>PO<sub>4</sub> the hair was physically disintegrated in a few days time, while in H<sub>2</sub>SO<sub>4</sub> after over 3 years the hair still retained its normal physical appearance. Examination of the liquid showed that approximately half of the original weight of the hair had been dissolved and was in proteose-peptone form in the proportion of about 40 per cent proteose to 60 per cent peptone. The sulfur content of both proteose and peptone was as high as that of the original hair. The filtrate was strongly levorotatory but contained no free cystine. The physically intact appearance of the undissolved portion suggests that the proteose-peptone fraction was derived from the medullary portion of the hair, the cortex being left largely intact.

**A Critical Study of the Sullivan Method for the Determination of Cystine.** BY JAMES C. ANDREWS AND KATHLEEN CRANDALL ANDREWS. *From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*

The cause of the increase in apparent cystine concentration of aging cystinuric urines as determined by the Sullivan method has been traced to atmospheric oxidation of urinary constituents which markedly inhibit the formation of color in the Sullivan method. Ascorbic acid is one of these constituents but is responsible for only a comparatively small proportion of the inhibiting effect.

Examination of a large variety of compounds to determine their ability to inhibit the formation of color from constant amounts of cystine has yielded the following results. The most

effective inhibitors are such reducing agents as ascorbic acid, adrenalin, hydroquinone, quinhydrone, pyrogallol and photographic developers in general, and  $\text{H}_2\text{S}$ . These almost completely inhibit color development, even when present in smaller amounts than the cystine. Almost as effective are a number of cyclic derivatives of cystine and of other amino acids.

A further class of substances, while effective only in larger amounts, is capable of producing erroneous results in the determination of cystine. These include the simple aldehydes, the sugars (both mono- and disaccharides), and various sugar derivatives such as glucuronic acid, its lactone, and certain of its conjugation products.

The observations of Lugg and of Rossouw and Wilken-Jorden concerning the interference of certain amino acids when present in large amounts are confirmed. These effects, however, are negligible compared to those mentioned above.

The inhibition curves of adrenalin and ascorbic acid form the basis of a satisfactory method of determining these substances in the absence of other such inhibitors.

### **A New Method for the Determination of Methionine in Proteins.**

By HARRY D. BAERNSTEIN. *From the Laboratory of Physiological Chemistry, University of Wisconsin, School of Medicine, Madison*

The calculation of methionine from volatile iodide recovered from HI digests of protein is open to valid objections. It has therefore been necessary to develop a second method for comparison. The new method is based upon oxidation of homocysteine produced from methionine.

0.5 gm. of protein is boiled 5 hours with 57 per cent HI containing 1 per cent  $\text{KH}_2\text{PO}_4$ . The digest is concentrated to 3.0 cc. and diluted to 25 cc. with air-free 4 per cent HCl. 10 cc. samples are oxidized with excess  $\text{KIO}_3$  and excess iodine titrated with  $\text{Na}_2\text{S}_2\text{O}_3$ . The iodine consumed is a measure of the cysteine present. The mixture is deaerated and made alkaline with  $\text{NH}_4\text{OH}$ . Homocysteine is thus liberated from thiolactone and oxidized by tetrathionate produced by the previous titration. Thiosulfate is formed and titrated with  $\text{KIO}_3$  after acidification. The specificity of the tetrathionate oxidation has been proved.



The results on fat-free proteins agree closely with those from volatile iodide. Ether and alcohol should be avoided in preparing proteins or else all traces subsequently removed.

This agreement between the two methods answers the objections raised regarding the assumption that all the volatile iodide comes from methionine.

**Echinochrome, Its Isolation and Composition.** BY ERIC G. BALL.

*From the Department of Physiological Chemistry, the Johns Hopkins University, School of Medicine, Baltimore*

The method previously described for isolation of the pigment of *Arbacia* eggs has been discarded. The echinochrome is partly decomposed by alkali treatment and yields a mixture difficult to purify. The pigment is extracted with acidulated alcohol, the solvent evaporated at room temperature, and the residue repeatedly extracted with petroleum ether to remove fats. Some pigment dissolves in the first fractions; subsequent extractions are colorless. The residue is dissolved in ethyl ether which, with evaporation, is gradually replaced by petroleum ether; on cooling, this yields a crystalline deposit of echinochrome. Recrystallization may be effected from benzene.

The compound melts fairly sharply at 220.5–221.0° (corrected), partial sublimation occurring. Tests for N, S, Cl, or P were negative. Microanalysis yielded the following values, C 54.00, 54.11; H 3.81, 3.93 per cent (courtesy of Dr. O. Wintersteiner). The formula  $C_{12}O_7H_{10}$  requires 54.11 per cent C and 3.79 per cent H. Molecular weight determinations by the camphor method were from 200 to 253, depending on the ratio of the mix; theoretical, 266. The pigment shows a color change from red to yellow, centered at about pH 6. The absorption spectra of the undissoiated red form exhibits two main peaks at  $\lambda$  490 and  $\lambda$  528 (courtesy of Mr. Davies). Echinochrome behaves like a polyhydroxy compound. Preliminary studies on the oxidation-reduction potential of the system indicate that it is more negative than any known naphthoquinone or anthraquinone system, though resembling in other respects the behavior of these compounds.

**Oxidation-Reduction Potentials of the Ascorbic Acid System.**

BY ERIC G. BALL. *From the Department of Physiological Chemistry, the Johns Hopkins University, School of Medicine, Baltimore*

This system is electromotively sluggish towards noble metal electrodes. So far a titration curve has been obtained only in phosphate buffer, pH 3.0, with ferricyanide as oxidizing agent. At other pH values, 1 to 5, electrode equilibrium is reached too slowly to obtain a complete titration curve. This holds true also for citrate and phthalate buffers at pH 3.0. If other oxidizing agents are used (quinone or iodine), attainment of electrode equilibrium is much more sluggish and unaffected by pH. The peculiar action of ferricyanide is ascribed to the decomposition of the ferrocyanide formed with liberation of some iron compound. This acts as a mediator to bring the electrodes more rapidly to equilibrium, the formation of which is maximum in phosphate buffer of pH 3.0. Cu or Fe salts, or methylene blue, depending on the pH, will also act as mediators. When these are used with the oxidizing agent quinone, results are obtained similar to those given by ferricyanide alone. The chemical reaction between ascorbic acid and the oxidizing agent is rapid. Therefore the ascorbic acid system is evidently intermediate in its behavior in the acid range to the dye systems, on the one hand, and such systems as fumarate-succinate, on the other.

The  $E'_0$ :pH curve at 30° from pH 1.0 to 7.0 can be described by the equation

$$E_h = E_0 + 0.03006 \log \frac{[S_o]}{[S_r]} + 0.03006 \log [K_1(H^+) + (H^+)^2]$$

where  $E_0 = +0.392$  and  $pK_1 = 4.1$ . The method of discontinuous titration was employed in obtaining results. The instability of the oxidant renders this method ineffective in the alkaline range and experiments in which the author's flow apparatus is employed are in progress.

**The Effect of Parenteral Injection of Amino Acids and Related Substances upon Creatine Formation and Storage in the Rat.**

BY HOWARD H. BEARD AND THOMAS S. BOGGESE. *From the Department of Biochemistry, Louisiana State University Medical Center, New Orleans*

The injection of 100 mg. of creatine, glycocyamine, arginine, histidine, glycine, alanine, serine, and valine for 1 day gave increases in muscle creatine of from 6 to 32 per cent above normal. At the end of 2 days these increases for the first four substances varied from 25 to 40 per cent, while the results with glycine at this time were negative. All of these increases have about disappeared at the end of 4 days after injection.

The injection of 200 mg. doses of arginine or histidine for 1 and 2 days gave from 30 to 40 per cent increase in muscle creatine. 200 mg. of arginine for 1 day gave an increase of 43 and 22 per cent, respectively, in stomach and liver creatine, while 100 mg. of histidine for 1 day gave a 19 per cent increase in stomach creatine. These were the only incidences of increased creatine formation in the stomach and liver in these experiments. 79 control and 189 experimental animals were used.

These results point to the following possibilities: (a) that creatine arises in the body as follows: arginine  $\rightarrow$  glycocyamine +  $\text{CH}_3$  (amino acids)  $\rightarrow$  creatine, (b) that no creatine storage occurs after 1 day following the injection of glycine, as compared to that obtained with other amino acids, (c) that creatine formation takes place chiefly in the muscles, and (d) that creatine is either a product of the exogenous amino acid metabolism or that these substances stimulate creatine formation.

**The Resolution of *dl*-Lysine.** BY CLARENCE P. BERG. *From the Biochemical Laboratory, State University of Iowa, Iowa City*

*d*-Camphoric acid was combined with 2 molecular equivalents of synthetic *dl*-lysine to form salts which were subsequently fractionated by repeated crystallization from methyl alcohol-water solutions. The more insoluble *d*-lysine *d*-camphorate was readily obtained in optically pure form. On dissolving this in water and adding hydrochloric acid, the bulk of the camphoric acid precipitated; the remainder was extracted with ether. Op-

tically pure *d*-lysine dihydrochloride,  $[\alpha]_D^{20} = +15.63^\circ$ , was obtained from the extracted filtrate by concentrating it to a syrup *in vacuo*, dissolving in alcohol, and precipitating with acetone.

The *l*-lysine component was prepared from the more soluble and optically less pure fractions by liberating the lysine with sulfuric acid, removing the *d*-camphoric acid and the sulfate, and recombining the lysine with *l*-camphoric acid prepared from *l*-borneol. The more insoluble *l*-lysine *l*-camphorate was then isolated in pure form by fractional crystallization and converted to the *l*-lysine dihydrochloride,  $[\alpha]_D^{20} = -15.79^\circ$ . When only the *d*-lysine is required, its yield can be increased by racemizing the optically impure *l*-lysine in the less active *d*-camphorate fractions and repeating the resolution procedure.

Hitherto no method for resolving *dl*-lysine has been available. In combination with the recent excellent and relatively simple synthetic procedure for preparing inactive lysine, the method described permits the preparation of *d*-lysine from a non-protein source. The method also makes possible a more accurate determination of the availability of *l*-lysine to the animal organism. Such studies are in progress.

**The Effect of Cholesterol and Choline on Liver Fat.** BY CHARLES H. BEST AND JESSIE H. RIDOUT. *From the School of Hygiene, University of Toronto, Toronto, Canada*

Previous experiments have shown that choline inhibits the deposition of cholesterol esters and neutral fat in the livers of rats which have been fed diets containing cholesterol. The present results demonstrate that under appropriate conditions choline accelerates the disappearance of these substances from the liver.

When a relatively small dose of cholesterol is given to rats in which the "cholesterol" fatty liver had been produced, the addition of choline to the diet causes a very definite fall in both the glyceride and cholesterol ester content of liver tissue.

When larger amounts of cholesterol are provided under similar conditions, there may be a fall in the glyceride fraction in both the control and the test animals during the early part of the experiment, while the esters are increasing. This effect is inde-

pendent of the action of choline in the control series and may be due to an "appropriation" of neutral fat by the cholesterol. The nature of this appropriation phenomenon has been investigated.

The effect of a "lipotropic" factor present in casein on the "cholesterol" fatty liver has been investigated.

**The Alimentary Fate of Hemin in Man.\*** BY FRANKLIN C. BING, FRANKLIN A. BENES, AND DONALD G. REMP. *From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland*

Three normal subjects were given 100 mg. of crystalline hemin daily, and the excretion of hematin was determined in the feces under controlled conditions. Only about 10 per cent of the ingested hemin was recovered in the stools. Hemin is therefore absorbed or broken down in the alimentary tract into simpler compounds, such as inorganic iron and porphyrins. The action of digestive fluids was studied *in vitro*, and only gastric juice was found to be capable of breaking down hematin. The action was slight and due to the acid. On the other hand, considerable destruction of hematin was observed to be effected by fecal bacteria, and it is suggested that the disappearance of hematin noted in the subjects can be attributed to this action.

Metabolism studies on one of the subjects showed that a slightly negative iron balance was converted to a positive balance when hemin was ingested. The extent of the retention indicates that in man the organic iron of hemin can be well utilized.

**The Distribution and Properties of the Chick Gizzard Factor.** BY H. R. BIRD, C. A. ELVEHJEM, AND E. B. HART. *From the Department of Agricultural Chemistry, University of Wisconsin, Madison*

In the course of vitamin B<sub>4</sub> studies involving the feeding of synthetic rations to chicks, a deficiency other than that due to lack of vitamin B<sub>4</sub> has been found to complicate the results. So far as external symptoms are concerned, the new deficiency is characterized only by lack of growth, but postmortem examination reveals characteristic lesions in the gizzard lining, apparently

\* Aided by Grant 209 from the Committee on Therapeutic Research, American Medical Association.

identical with those described by Dam and Schönheyder.<sup>1</sup> However, the factor involved is not fat-soluble, and hence must be distinct from the antihemorrhagic factor studied by the above investigators. Pork lung, liver, and kidney have been found to be good sources of the factor. Pork heart and brain, crude casein, and wheat are intermediate sources; and yeast, alfalfa, dried grass, yellow corn, peanuts, egg yolk, corn oil, wheat germ oil, and soy bean oil are relatively poor sources.

Experiments on heat stability show that the new factor is destroyed in a grain ration either by dry heat at 120° for 24 hours or by autoclaving at 15 pounds pressure for 5 hours. However, the factor as it exists in lung tissue is considerably more stable, being quite resistant to either of the above methods of treatment.

The factor is not extracted from lung tissue by ether, by 95 per cent, or by 50 per cent alcohol, and is extracted only to a very limited extent by hot water. Attempts to obtain it in solution have thus far been unsuccessful.

**Influence of Delayed Resorption of the Protein Hormones.** By FRITZ BISCHOFF AND L. C. MAXWELL. *From the Chemical Laboratory, Santa Barbara Cottage Hospital, Santa Barbara*

Following the observations originally made in this laboratory that the qualitative and quantitative response to pituitary gonadotropic extracts and to insulin was markedly changed by administering these substances in combinations with protein precipitants or adsorbents, a systematic investigation of the combinations of various hormones and various agents was made. The proteins may be precipitated on the acid side of the isoelectric point by such substances as tannic acid; they may be adsorbed by  $\text{Zn}(\text{OH})_2$ , etc., at a pH more nearly neutral. Finally, they may form insoluble compounds with the basic proteins, which break up very slowly at the pH of the body. The effect is enhanced by excess of precipitant.

Tested upon rats, insulin tannate shows a marked augmentation in effect over regular insulin, as indicated by a frequency curve for incidence of shock. In rabbits sensitized to insulin dosage no augmentation in hypoglycemic effects but merely delay is noted

<sup>1</sup> Dam, H., and Schönheyder, F., *Biochem. J.*, **28**, 1355 (1934).

in contrast to rabbits unaccustomed to insulin dosage. The combination of insulin with thymus histone is so insoluble that the action is delayed over 12 hours. The physiologic effect of prolan, which to date must be classed as a protein hormone, is not affected by tannic acid,  $\text{Zn}(\text{OH})_2$ , or thymus histone. None of these substances was found to precipitate prolan quantitatively.

### **Muscle Glycogen Content of Rats Fasted Twenty-Four Hours.**

BY N. R. BLATHERWICK, PHOEBE J. BRADSHAW, AND SUSAN D. SAWYER. *From the Biochemical Laboratory of the Metropolitan Life Insurance Company, New York*

It has been reported that the muscle glycogen content of rats fasted 24 hours varies directly with the carbohydrate content of the preceding diet. Our experience indicated that muscle glycogen is, probably, not so easily influenced. We have, therefore, reinvestigated this question, using methods designed to give maximum values for glycogen. The diets used were calculated to contain 77.2, 65.9, and 54.0 per cent carbohydrate. Twelve male rats were placed on each of the diets at ages varying from 27 to 44 days, and were killed after they had eaten the diets for periods varying from 48 to 59 days. The following values for the *extensor* thigh muscles were obtained:  $564 \pm 21.1$ ,  $489 \pm 17.5$ , and  $605 \pm 9.7$  mg. per 100 gm. The corresponding values for the *flexor* leg muscles were  $539 \pm 13.0$ ,  $503 \pm 10.2$ , and  $549 \pm 14.6$  mg. per 100 gm. These results show that the carbohydrate content of the diet preceding the fast did not affect the glycogen content of the muscles.

### **Some Observations on Follicle-Stimulating Hormone Obtained from Urine of Women in and past the Menopause.** BY RICHARD J. BLOCK, ERWIN BRAND, MEYER M. HARRIS, AND L. E. HINSIE. *From the Departments of Chemistry, Internal Medicine, and Clinical Psychiatry, New York State Psychiatric Institute and Hospital, New York*

The concentrates were prepared by the  $\text{Al}(\text{OH})_3$  method and somewhat further purified.<sup>2</sup> Mixed batches equivalent to 500 liters per batch were used in the preparations of follicle-stimulating

<sup>2</sup> Brand, E., Block, R. J., Harris, M. M., and Hinsie, L. E., *Proc. Soc. Exp. Biol. and Med.*, **33**, 360 (1935).

hormone powder. 300 mg. of powder produced no ill effects in immature female rats from our own and another colony, but 10 mg. were decidedly toxic to immature female mice, possibly on account of the high salt content of the preparation.

Follicular stimulation is observed in ovaries weighing up to 100 mg. Throughout this range the ovarian response is approximately a straight line function of the amount of follicle-stimulating hormone. Increased amounts of the hormone may produce luteinized ovaries weighing up to 200 mg., but still greater amounts may cause involution, the ovaries being largely luteinized. These observations are consistent with current views on the mechanism of follicle-stimulating hormone action.<sup>2</sup>

Pregnancy urine (antuitrin S) has practically no synergistic action when injected together with follicle-stimulating hormone powder prepared by the  $\text{Al}(\text{OH})_3$  method. This is in contrast to the reported behavior of other follicle-stimulating hormone preparations, including alcohol precipitates from menopause and castrate urine.

Addition of Al or Zn salts<sup>4</sup> did not increase the potency of our follicle-stimulating hormone concentrates which were practically free from these metals.

**Nitrogen and Creatine Metabolism in Relation to Environmental Temperature and Thyroid Function.** BY MEYER BODANSKY AND VIRGINIA B. DUFF. *From the Department of Pathological Chemistry, School of Medicine, University of Texas, and the John Sealy Hospital, Galveston*

The increased output of nitrogen and of creatine and creatinine in rats exposed to cold approaches levels attained in thyroid- and thyroxine-treated animals. In untreated rats the changes in metabolism are related to hyperplasia of the thyroid. The combined effect of exposure to cold and the administration of thyroid substance on creatine metabolism is no greater than the effect of thyroid alone.

Compared to the relative constancy of creatine-creatinine excretion under ordinary conditions are the marked fluctuations

<sup>2</sup> Cf. Fevold, H. L., Hisaw, F. L., and Greep, R., *Am. J. Physiol.*, **114**, 508 (1936).

<sup>4</sup> Cf. Maxwell, L. C., *Am. J. Physiol.*, **110**, 458 (1934-35).



in hyperthyroidism, whether induced by exposure to cold or the administration of thyroid substance. The results suggest an unsteady state of metabolism and are considered to be a striking example of disturbed homeostasis.

The predictable response of the normal rat to a low thermal environment has been made the basis of a test of thyroid function in thyroparathyroidectomized rats.

**The Concentration and Properties of Vitamin H.** BY LELA E. BOOHER. *From the Department of Chemistry, Columbia University, New York*

A concentrate of vitamin H free from both vitamin B ( $B_1$ ) and vitamin G (lactoflavin) has been prepared from rice polishings. The concentrate is active in promoting growth in young rats in doses of less than 1 mg. per day. Relative to vitamin B ( $B_1$ ), vitamin H is heat-stable; it is relatively stable in acid or neutral media, but is readily destroyed by alkalis. Vitamin H, like vitamin G, is essential for growth and for the maintenance of a normal condition of the skin.

**Further Experiments upon the Excretion of Ascorbic Acid in the Urine Following Ether Anesthesia.** BY DONALD E. BOWMAN AND EDWARD MUNTWYLER. *From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland*

It was recently reported by us<sup>6</sup> that following ether anesthesia in the dog the urinary excretion of ascorbic acid as determined by titration with 2,6-dichlorophenol indophenol is markedly increased. This finding has subsequently been repeatedly observed with dogs and also with rats and guinea pigs, the only other animals studied so far. The excess excretion, which amounts to a 10- to 15-fold increase in the case of the dog but which is somewhat less in the case of rats and guinea pigs, is only of short duration. That is, the excretion falls to the normal level or slightly below in the second 24 hour urine collection following the anesthesia.

To demonstrate that the titration value with 2,6-dichlorophenol indophenol represents ascorbic acid, a dog was subjected to eight

<sup>6</sup> Bowman, D. E., and Muntwyler, E., *Proc. Soc. Exp. Biol. and Med.*, **33**, 437 (1935).

successive 2 hour periods of anesthesia at intervals of from 4 to 8 days. The urine was collected following each anesthesia and was treated with lead acetate in alkaline solution. The precipitate which formed was separated by centrifuging and dissolved in dilute acetic acid. The lead was then precipitated with hydrogen sulfide. The filtrates so obtained were standardized with 2,6-dichlorophenol indophenol. Such preparations, when given orally to guinea pigs in amounts equivalent to 1.55 mg. of ascorbic acid per 100 gm. of body weight per day, prevented the development of scurvy in animals receiving Sherman's basal scurvy-producing diet.

**Canine Cystinuria. III.** BY ERWIN BRAND AND GEORGE F. CAHILL. *From the Department of Chemistry, New York State Psychiatric Institute and Hospital, and from the Squier Urological Clinic of the Presbyterian Hospital, New York*

A case of cystinuria in a pedigreed male Irish terrier was recently described.<sup>6</sup> The dog excreted in the urine large amounts of neutral S (54 per cent of the total S) and of cystine. However, cystine accounted for only 30 per cent of the neutral S, indicating the excretion of considerable quantities of another S compound, as yet unidentified. Unfortunately, the dog was not available for continued metabolic observations.

No further case of cystinuria was discovered among those relatives of the dog, which we were able to trace and to investigate. However, we succeeded in mating the original cystinuric dog with two half-sisters, and with an unrelated female of the same breed. Three litters, a total of fifteen pups, were obtained. None of these pups has cystinuria, but we expect that interbreeding will reestablish the disease in the next generation.

Some evidence on the hereditary nature of canine cystinuria is already available. Through the courtesy of Dr. Morris another Irish terrier was obtained in which cystinuria was established. Investigation of this animal's pedigree showed it to be a son of a male litter mate of the original cystinuric dog.

It seems to us that valuable information, alike from the standpoint of genetics, metabolism, and calculus formation, could be gained from a study of cystinuric animals.

<sup>6</sup> Morris, M. L., Green, D. F., Dinkel, J. H., and Brand, E., *North Am. Vet.*, **16**, 16 (1935).

We are indebted to Dr. Morris and Mr. Green of the Raritan Hospital for Animals for their continued interest and cooperation.

**Some Recent Studies on Fat Deficiency.** BY W. R. BROWN AND GEORGE O. BURR. *From the Department of Botany, University of Minnesota, Minneapolis*

A composite growth curve for over 200 fat-deficient rats is practically identical with those curves previously published by Burr and coworkers on small groups.

It is now evident that relatively impure diets may be used for the production of typical fat deficiency symptoms. When crude casein is substituted for purified casein, the weight at time of plateau is slightly higher and decline and death are postponed. However, hematuria, scaly feet, and scaly and necrotic tails still occur to almost the same extent. The substitution of fresh skim milk for casein and of potato starch for part of the sucrose produces animals similar to those fed crude casein and sucrose. Butter-milk lipids which have appreciable quantities of highly unsaturated, long chain fatty acids are non-curative when fed to fat-deficient rats. Yeast oil is likewise ineffective.

An observation that scaliness decreases during the summer months was followed by controlled humidity experiments which have shown that low humidity favors development of scaliness, while high humidity reduces it.

**On Carbohydrate Metabolism in Adrenalectomized Animals.**

BY MARY V. BUELL, IAN A. ANDERSON, AND MARGARET B. STRAUSS. *From the Chemical Division, Department of Medicine, the Johns Hopkins Hospital and the Johns Hopkins University, Baltimore*

The fate of ingested *d*-lactic acid in adrenalectomized rats has been studied in a variety of experimental conditions. The slow absorption of *d*-lactic acid found for rats in adrenal insufficiency determined the amounts of lactic acid which were given to the other groups.

Certain adrenalectomized rats were allowed to go into insufficiency; others were kept in good condition by oral administration either of Grollman's charcoal adsorbate or Rubin-Krick's salt solution. Regardless of their treatment all adrenalectomized rats after

a 24 hour fast showed lower stores of carbohydrate in blood, liver, and muscles than did normal fasting rats. The factor permitting conversion of endogenous protein into carbohydrate was not supplied by either therapy.

Ingested *d*-lactate accumulated in the blood of animals in adrenal insufficiency and was only slowly converted into liver glycogen, thus confirming the hypothesis of Buell, Strauss, and Andrus. The blood sugar of these lactate-fed animals was low. Adrenal-less rats in good condition owing to salt therapy also failed to make normal use of *d*-lactate. Their blood sugar was somewhat higher than that of animals in adrenal insufficiency and their liver glycogen slightly lower. Adrenalless animals protected by charcoal adsorbate made almost normal use of *d*-lactic acid, converting it into liver and muscle glycogen and blood sugar.

Apparently the many errors in carbohydrate metabolism resulting from adrenalectomy do not necessarily cause the rapid death of the animal which is protected by salt from excessive loss of water and electrolytes.

**A Study of the Influence of Heavy Water upon Amylase Formation during the Sprouting of Barley.** BY M. L. CALDWELL AND S. E. DOEBBELING. *From the Department of Chemistry, Columbia University, New York*

Amylase activity increases markedly during the germination of many grains and there is evidence of the presence of at least two distinct amylases in malted barley. As water is involved in the changes which take place, it seemed of special interest to study the influence of heavy water upon the relationship which exists between the sprouting of barley and the generation of amylase. The study includes a comparison of the amylase activity of barley grains which have been allowed to sprout in different concentrations of heavy water and in ordinary water. The comparisons were made at definite time intervals during the course of germination and at what appeared to be the same stages of germination. The concentrations of heavy water studied were 100, 50, 10, and 1 per cent. The higher concentrations of heavy water retard germination and amylase generation. Whether the two amylases are similarly affected is being actively investigated.

**Creatine and Creatinine Metabolism in Infancy and Childhood.**

By RUTH CATHERWOOD AND GENEVIEVE STEARNS. *From the Department of Pediatrics, College of Medicine, State University of Iowa, Iowa City*

This report summarizes the urinary creatine and creatinine data obtained from 73 24 hour studies of 22 infants from birth to 1 week of age and 409 3 day studies of 62 infants from 2 weeks to 1 year of age, together with 314 studies of creatinine excretion of 45 older children.

Excellent correlations were obtained between creatinine: weight ( $0.9056 \pm 0.0055$ ) and creatinine: length ( $0.893 \pm 0.0065$ ) during infancy. These are interpreted as additional proof in the living child of the anatomical findings that the musculature and the skeleton both remain a constant proportion of the body weight throughout the 1st year. From the studies of older children it appears that this relative proportion of muscle to body weight is maintained until the 3rd year, the quantity of musculature increasing rapidly thereafter until adult proportions are reached.

Each infant studied always excreted creatine. The amounts increased throughout infancy, although neither weight nor age appears to be a major factor, nor could any relation be observed between creatine excretion and any phase of nitrogen metabolism. In infancy, both creatine and protein intake are apparently minor factors in determining the level of creatine output. Experiments with older children suggest that the increased creatinuria observed after a sharp increase in protein intake may be a transitory phenomenon. The data obtained indicate that the maximum endogenous creatine excretion is reached in early childhood, and that this level may be maintained until adolescence.

**Influence of Liver Damage on the Blood Lipids.** By ALFRED CHANUTIN AND STEPHAN LUDEWIG. *From the Biochemical Laboratory, University of Virginia, University*

A patient with liver damage and xanthomatosis was studied. The free cholesterol and phosphatide phosphorus of blood plasma were tremendously increased. The cholesterol esters and neutral fat were either absent or present in small amounts. A "fat-free" diet plus administration of choline, betaine, thyroid, liver extract,

and insulin failed to reduce the abnormal values and distribution of the lipids in the blood plasma.

Liver damage in rats produced by partial hepatectomy or by ligation of the bile duct resulted in elevated total cholesterol values of the blood plasma and increased ratios of free to total cholesterol. The phosphatide phosphorus values were proportional to the free cholesterol.

**Evaluation of the Antianemic Potency of Liver Extract by the Jacobson Method of Bioassay.** BY GUY W. CLARK AND ALICE M. COENE. *From the Lederle Laboratories, Pearl River, New York*

The content of the antianemic substance present in different liver extracts has been estimated by the guinea pig method of bioassay. Although the method may not be specific, reproducible results are obtained.

**Studies on the Mechanism of Metabolic Stimulation by Dinitro-, Dihalo-, and Trihalophenols.** BY G. H. A. CLOWES AND M. E. KRAHL. *From the Lilly Research Laboratories, Indianapolis*

Study of the effects produced by nitro- and dinitrophenols on the oxygen consumption of sea urchin eggs and on the oxygen consumption and anaerobic fermentation of yeast has indicated that the action of these compounds on metabolism is not attributable to reduction and oxidation of the nitro groups, and that the phenolic hydroxy group is indispensable. In support of this view, it was found that the oxygen consumption of sea urchin eggs could be stimulated by phenols having no nitro and only halogen substituents in the benzene ring.

When these same halogen compounds were administered orally or intravenously to animals, no significant increases in respiratory rate or body temperature were observed. However, under suitable experimental conditions, the oxygen consumption of excised mammalian tissues and the oxygen consumption and anaerobic fermentation of yeast were increased by dihalo- and trihalophenols, the increases so produced being comparable to those effected by dinitrophenols.

The results are of interest in relation to the cellular processes which may be involved in the stimulation of oxidation and fermentation.

**The Spontaneous Decomposition of Cystine Dimethyl Ester.**

By ROBERT D. COGHILL. *From the Sterling Chemistry Laboratory, Yale University, New Haven*

Although the hydrochloride of cystine dimethyl ester is stable for long periods of time, the free ester decomposes spontaneously at room temperature, about one-third of the sulfur crystallizing out as free sulfur. In addition to the sulfur, ammonium sulfate and *dl*-alanine anhydride have also been identified as decomposition products, and three other substances of unknown nature have been obtained.

It is believed that the ester first condenses to cystine anhydride, which then breaks down with the liberation of sulfur and hydrogen sulfide, the latter substance possibly serving as the agent which reduces the resulting methylene compound to *dl*-alanine anhydride. The above reaction, however, is only one of probably several competing reactions which produce an amorphous solid and an oil boiling at 155° at 17 mm., each of which contains both nitrogen and sulfur.

**On the Relation between the Solubility in Different Solvents of Amino Acids, Peptides, and Certain Related Substances.**

By EDWIN J. COHN AND THOMAS L. McMEEKIN. *From the Department of Physical Chemistry, Harvard Medical School, Boston*

The analysis of the solubility of amino acids, peptides, and certain related substances has been extended not only to water and ethanol, but to acetone, formamide, and higher alcohols. Although a few exceptions have been noted, presumably related to observed crystal lattice energies, several rules for these classes of molecules have emerged, which may be simply stated as follows: (1) Each additional CH<sub>2</sub> or phenyl group decreases solubility in water, and increases solubility in alcohols. (2) Each additional hydroxyl, carboxyl, amide group, or peptide linkage, decreases solubility in both water and alcohol. (3) Dipolar ionization generally increases solubility in water,  $N_O$ , but diminishes solubility in alcohol,  $N_A$ . (4) The quantity,  $\log .N_A / N_O$ , which for relatively insoluble solutes yields the change in free energy with change in solvent, is diminished by 2.7 by the formation of an ionized di-pole, regardless of the distance between the ammonium and carboxyl groups. It is diminished far more, however, if the

number of such groups is increased. The comparable relation between formamide and water is 2.0 and between acetone and water 3.4. (5) Each  $\text{CH}_2$  group in the paraffin chain increases the logarithm of the solubility ratio between formamide and water by 0.23. The influence of the  $\text{CH}_2$  group is greater the less polar the solvent, being 0.44 for methanol, 0.49 for ethanol and acetone, and 0.53 for butanol and heptanol, water always being taken as standard state.

These rules would appear applicable to the separation and purification of molecules containing the groups thus far investigated, as well as to the interpretation of the behavior, on the one hand, of multipolar peptides, proteoses, and proteins, and, on the other, of lipopeptides.

**The Conversion of Hexoses and Trioses to Glucose in the Liver of the Rat.** BY CARL F. CORI AND WILLIAM M. SHINE. *From the Department of Pharmacology, Washington University School of Medicine, St. Louis*

Liver slices of fasted rats were incubated in oxygenated bicarbonate-Ringer's solution at  $37^\circ$  and pH 7.5. To one or two equal portions of liver slices various substrates were added, while a third portion was incubated without added substrate. Fermentable sugar was determined after hydrolysis in  $\text{N}$  sulfuric acid. If sugars other than glucose were present after hydrolysis, they were determined by separate methods, so that the actual change in glucose content could be calculated. The following order in the rate of conversion to glucose was observed, fructose being taken as 100: dihydroxyacetone 71, glyceraldehyde 58,  $\alpha$ -glycerophosphate 56,  $\beta$ -glycerophosphate 31, glycerol 30, galactose 20, mannose 9. Anaerobiosis or the addition of cyanide prevented the conversion of these substances to glucose. A large portion of added fructosemonophosphate was converted in a few minutes to glucosemonophosphate, followed by a liberation of glucose and inorganic P due to phosphatase activity. Fructosediphosphate was dephosphorylized at a much slower rate than the monophosphate. A liver extract almost free of phosphatase activity was prepared which yielded, upon addition of fructosemonophosphate, in 10 minutes an equilibrium mixture of 50 per cent fructose- and glucosemonophosphate.



**Catabolism of Straight and Branched Chain Amino and Fatty Acids in the Normal and Phlorhizinized Dog.** BY RALPH C. CORLEY AND JOHN A. LEIGHTY. *From the Laboratory of Biochemistry, Department of Chemistry, Purdue University, Lafayette*

The importance of biochemical substances containing branched aliphatic chains makes desirable more knowledge about the metabolism of compounds containing methyl and other alkyl radicals. We have been interested also in extending earlier observations of one of us (R. C. C.) on the catabolism of synthetic amino acids. A number of synthetic amino acids have been administered to the completely phlorhizinized dog, and also to the normal dog maintained in nitrogen balance.

The following conclusions have been drawn tentatively for the compounds studied. Amino acids with straight chains yield their nitrogen as urea in the normal dog more completely than do amino acids with branched chains, particularly than do those in which a methyl group is on the same carbon as the amino group. Extra glucose formation in the completely phlorhizinized dog is not that which would be expected if, in their catabolism, these compounds were converted to fatty acids containing 1 carbon atom less. For example, *dl*- $\alpha$ -aminobutyric acid has not been found to be a sugar former, although propionic acid is so considered.

Studies with fatty acids are in progress.

**An Apparatus for Milking Small Laboratory Animals, and the Composition of Stock Rat Milk.** BY WARREN M. COX, JR., AND ARTHUR J. MUELLER. *From the Research Laboratory, Mead Johnson and Company, Evansville, Indiana*

A micro-milking apparatus has been developed, and has proved satisfactory for milking rats and guinea pigs. Alternating negative pressure is obtained from a water pump connected with a magnetic switch cut-off, and a relay adapted to give adequate lag in operation. 7 inches (Hg) of negative pressure, and twenty-five pulsations per minute is optimal for milking rats, as compared to 16 inches and forty-four pulsations used in cow-milking machines. The large end of a small rubber catheter was employed as a teat cup.

The rat mother is separated from her young for 12 hours or

less before milking. Large variations in the volume of milk from an individual rat may be expected, but, on the average, the following volumes can be obtained from stock mothers: 8th day of lactation, 0.5 cc. per rat; 12th day, 1.0 cc.; 17th day, 1.5 cc.; 24th day, 0.7 cc. From guinea pigs, much larger volumes are readily obtained.

The percentage composition of rat milk at different stages of lactation was determined. The following figures are averages of all determinations: fat, 14.8 per cent; protein, 11.3 per cent; carbohydrate, 2.9 per cent; ash, 1.5 per cent; solids, 31.7 per cent;  $d_{25}^{25}$  1.047; ratio of lactalbumin to casein, 1:12; pH (electrometric), 6.5 to 6.6. The extracted fat had a saponification number of 221 and an iodine number of 39.9. Ash analyses are in progress. The size of the fat globules is approximately the same as in cow's milk. Buffer curves show 170 cc. of 0.1 N acid (as compared to 65 cc. for cow's milk) to 100 cc. of rat milk are required to lower the pH to 4.0.

#### **Amino Acid Content of Staple Foods.** BY FRANK A. CSONKA.

*From the Protein and Nutrition Research Division, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington*

The total nitrogen extractable from whole wheat flour by solvents is lower than that previously found for patent flour.

Only 83 per cent of the total nitrogen was removed by solvents, and an additional 10 per cent was recovered by hydrolyzing the residue with 20 per cent hydrochloric acid. Determination of cystine, tryptophane, tyrosine, arginine, and lysine in whole wheat flour gave results ranging from 30 to 50 per cent lower than the corresponding figures found for patent flour. These findings are of significance when wheat is used to supplement amino acid deficiencies in a faulty diet.

#### **The Acid-Base Balance of the Blood Serum in Hyperthermia.**

BY WAYNE H. DANIELSON AND ROBERT M. STECHER. *From the Departments of Biochemistry and Medicine (City Hospital Division), School of Medicine, Western Reserve University, Cleveland*

The changes in the total acid-base balance of human blood as a

result of artificial fever have been studied. Since it has been observed that the feeling of fatigue and depression following a hyperthermia treatment can be largely overcome if the patient drinks a dilute salt solution during the treatment, the effect of drinking a 0.6 per cent NaCl solution on the acid-base balance of the blood during fever has also been studied.

The serum acid-base changes observed as a result of either 2 or 4 hours fever at 40° when water was given *ad libitum* were an elevation of the pH, a fall in the CO<sub>2</sub> tension, and a decrease in the bicarbonate, chloride, inorganic phosphorus, and total base concentrations. The change in the serum protein concentration was not significant. The decrease in the total determined acid concentration was greater than that of the total base, so that there was an increase in the undetermined acid (presumably sulfates and organic acid).

The drinking of a 0.6 per cent NaCl solution during similar periods of fever produced no significant difference in the changes in the pH, CO<sub>2</sub> tension, and the bicarbonate, protein, inorganic phosphorus, total determined acid, and undetermined acid concentrations from those observed when water was drunk. However, the chloride concentration increased above the control level and there was not as great a decrease in the total base concentration at the end of the 4 hour fever periods.

### **The Vitamin G Complex in Rat Dermatitis and Human Pellagra.**

BY W. J. DANN. *From the Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina*

György has shown that a characteristic dermatitis appears regularly in rats fed on a diet devoid of all water-soluble vitamins, to which vitamin B and lactoflavin are added. The dermatitis produced is similar to that seen by Goldberger, which has been thought to be analogous to pellagra in the human being; it is curable by an accessory factor named vitamin B<sub>6</sub>.

Rats maintained on the György regimen develop the dermatitis when kept in the dark as quickly as in the light; the dermatitis is thus not analogous to the dermatitis of the exposed areas occurring in human pellagra, which is only seen after exposure to the sun.

A liver preparation containing vitamin B<sub>6</sub> has been found in

preliminary clinical trials at Duke Hospital to be devoid of pellagra-preventive activity. Similarly, preliminary trials show lactoflavin to be devoid of pellagra-preventive activity, suggesting that neither of the recognized constituents of the vitamin G complex is identical with the P-P factor of Goldberger.

Rats maintained on a diet devoid of water-soluble vitamins with the addition of vitamin B, lactoflavin, and vitamin B<sub>6</sub> grow at a rate far below the normal. Replacement of the lactoflavin and vitamin B<sub>6</sub> by 0.5 gm. daily of whole liver or dried yeast causes an immediate large increase in the rate of growth, indicating that the vitamin G complex contains other constituents in addition to lactoflavin and vitamin B<sub>6</sub>.

**Leucopenia and Anemia in the Monkey Resulting from Dietary Deficiency.\*** BY PAUL L. DAY, WILLIAM C. LANGSTON, AND CARROLL F. SHUKERS. *From the School of Medicine, University of Arkansas, Little Rock*

Young rhesus monkeys given a diet composed of whole wheat, polished rice, purified casein, Osborne and Mendel salt mixture,<sup>7</sup> salt, cod liver oil, and orange, developed a marked anemia and leucopenia which terminated fatally within 26 to 93 days. The substitution of 0.01 gm. of cevitamic acid daily for the orange did not significantly alter the course of the disease. The deficient diet supplemented with 10 gm. of dried brewers' yeast daily supported normal growth and development, and maintained a normal blood picture over a period of 400 days. 2.5 gm. of yeast proved inadequate, however. 2 gm. of extralin (a liver-stomach preparation) daily likewise promoted normal growth and development for a period of 400 days.

**Sweat.** BY D. B. DILL, CORNELIUS A. DALY, AND A. V. BOCK. *From the Fatigue Laboratory, Morgan Hall, and the Department of Hygiene, Harvard University*

Our studies of sweat secretion in the dry heat of Boulder City indicated that the concentration of NaCl shows individual variations and decreases during acclimatization. Experiments with

\* Aided by grants from the Committee on Scientific Research, American Medical Association.

<sup>7</sup> Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

a similar outcome have been made in the more humid heat of the Youngstown steel mills. Heat cramps, which depend on excessive salt depletion, occur most commonly in the first hot spells of the season, or in men returning to work after a lay off.

Sweat produced at a room temperature of 40–50° in the winter season varies extraordinarily in composition. One man put out a sweat containing from 14 to 27 mm of NaCl per liter in rest and from 28 to 39 in work. In another man, under similar conditions, the corresponding values were 42 to 69 and 70 to 77 mm, respectively. The second man had had a concentration of from 12 to 30 mm after acclimatization at Boulder City. We conclude that the following factors are important in determining the NaCl content of sweat: (a) degree of acclimatization, (b) air temperature, (c) intensity of work, and (d) individual characteristics.

#### **A Study of the Porphyrins Excreted in Congenital Porphyrinuria.**

By KONRAD DOBRINER, SYLVIO LOCALIO, AND WILLIAM H. STRAIN. *From the Departments of Medicine and Biochemistry, The University of Rochester School of Medicine and Dentistry, Rochester, New York*

The porphyrins excreted in the urine and feces of two cases of congenital porphyrinuria, both with photosensitivity, have been studied. In the urine coproporphyrin I and uroporphyrin I are present. The feces of both patients contain coproporphyrin I and at least two other porphyrins. Acid numbers and absorption spectra indicate that these two porphyrins belong to the proto- and deuteroporphyrin groups, respectively. Work is in progress toward their complete identification. The amount of these uncharacterized porphyrins is too great to originate from the diet and they must be regarded as metabolic products.

The porphyrin-containing substances of biological origin, *e.g.* hemoglobin, cytochrome, chlorophyll, etc., are all made up of Type III porphyrins. The normal urinary porphyrin, excreted at a daily level of 30 to 80 micrograms, is coproporphyrin I, a Type I porphyrin. In the congenital porphyrinurias examined the level of excretion is very much higher. To reconcile this dualism of types of porphyrins, the Type I porphyrins may originate from physiological entities not yet characterized or by

disproportionation in the biological synthesis of Type III porphyrins. From this latter point of view the Type I porphyrins may be assumed to originate *in vivo* as a normal by-product in the production of Type III porphyrins in a manner analogous to *in vitro* syntheses. Thus congenital porphyrinuria may be regarded as a metabolic condition where marked disproportionation in favor of Type I porphyrins exists.

**An Analysis of the Absorption Spectra of Hemoglobin.** By  
DAVID L. DRABKIN. *From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*

The complex curves, which represent the absorption spectra of most of the common hemoglobin derivatives, may be analyzed into component bands.<sup>8</sup> All of the ultra-violet bands and one or two in the visible region belong presumably to a single series, which may be roughly defined by  $n = (\nu \times 10^{-2})/60$  or  $n = (\nu \times 10^{-2})/40$  where  $n$  is a simple integer and  $\nu$  the wave number. These bands are deduced to be related to the same fundamental disturbance in the molecule incident to the absorption of energy.

The so called  $\alpha$  bands of the gaseous derivatives of hemoglobin,  $\text{HbO}_2$ ,  $\text{HbCO}$ , and  $\text{HbNO}$ , do not belong to the above series. The differences in the visible absorption spectra of these derivatives are accounted for by their graphical analysis into two similar curves, whose peaks ( $\alpha$  and  $\beta$ ) are separated by different distances. These points are of importance: The true positions of the peaks can be obtained only after analysis, since displacements may occur in the additive spectrum. The greater proximity of the  $\alpha$  and  $\beta$  bands and relatively greater intensity of the  $\gamma$  band in  $\text{HbCO}$  than in  $\text{HbO}_2$  may be related to the greater affinity of hemoglobin for CO. This may lead to significant deductions.

A working hypothesis, suggested by the analysis and used, thus far, only for guidance, is that the hemoglobin spectra are largely an expression of the spectrum of Fe in coordinated compounds. A study of the spectra of hematoporphyrin and of ferricyanide does not oppose such a hypothesis.

<sup>8</sup> Drabkin, D. L., *Proc. Soc. Exp. Biol. and Med.*, **32**, 456 (1934).

**Comparative Estimations of Free Cholesterol in Blood by the Pyridine Cholesteryl Sulfate and the Cholesterol Digitonide Methods.** BY I. J. DREKTER, ALBERT E. SOBEL, AND SAMUEL NATELSON. *From the Achelis Laboratory, Lenox Hill Hospital, and the Pediatric Research Laboratory of The Jewish Hospital of Brooklyn, New York*

Comparative determinations were made of the "free cholesterol" in normal sera by the pyridine cholesteryl sulfate and by the cholesterol digitonide methods. The free cholesterol values (expressed in terms of the total cholesterol) were from 6 to 10 per cent by the new procedure and 25 to 35 per cent by the digitonide method. The validity of the new method for the estimation of "free cholesterol" was shown by the quantitative recoveries of cholesterol, added to the lipid extracts. The question that some form of cholesterol may not be determined was answered by the fact that total cholesterol values, subsequent to saponification, were even slightly higher by the new method than by the digitonide procedure. The higher "free cholesterol" values by digitonin may be due to the breaking of the chemical bond or the precipitation of some form of combined cholesterol other than the fatty and ester type.

The "free cholesterol" values of the clotted red cells were compared similarly. The values by the new method ranged from 62 to 84 per cent and 71 to 90 per cent by the digitonin method. It is concluded therefore that most of the free cholesterol in normal blood is in the red cells.

**Raman Spectra of *l*-Ascorbic Acid and Its Sodium Salt.** BY JOHN T. EDSALL. *From the Department of Physical Chemistry, Harvard Medical School, Boston*

Raman spectra often yield valuable evidence in the study of molecular structure. This method has already been applied by us to the ionization of the carboxyl group in fatty acids and amino acids. Recently Kumler and Daniels have pointed out that the first dissociation constant of ascorbic acid is larger than would be expected from the generally accepted chemical formula, according to which the dissociating group is enolic. Raman spectra were studied in the hope of throwing further light on the problem. Both *l*-ascorbic acid (pH 2) and its sodium salt (pH 5.1), in

aqueous solution, give strong Raman spectra, each spectrum containing about fifteen lines. The majority of the Raman frequencies are identical in the acid and in the salt; but one very broad powerful line—the most intense in the whole spectrum—shifts from a frequency of  $1703\text{ cm.}^{-1}$  in the acid to  $1590$  in the salt, while remaining very intense in both. This is in marked contrast to the behavior of carboxylic acids in water, which give rise to a strong line near  $1720\text{ cm.}^{-1}$  in the free acid, which apparently vanishes completely on ionization of the carboxyl group. The strong line in ascorbic acid may well be due to a  $\text{C}=\text{C}$  or a  $\text{C}=\text{O}$  linkage, or both. Final interpretation must await the study of related compounds, now being investigated, but the results already obtained appear compatible with the generally accepted formula for ascorbic acid.

**Phenolic Substances of Urine.** BY BEATRICE G. EDWARDS.

*From the Department of Biochemistry, University of Oklahoma Medical School, Oklahoma City*

Urinary substances reacting with the Folin-Denis and diazotized *p*-nitroaniline phenol reagents have been fractionated by Lloyd's reagent, lead subacetate, barium hydroxide, alkaline mercury, and by ether extraction. Original urine gave high Folin-Denis values. The tungstomolybdate to *p*-nitroaniline ratios were approximately 4, but varied.

Lloyd's reagent removed uric acid quantitatively, lowered the phenol values more than one-half, and changed the tungstomolybdate to *p*-nitroaniline ratio to approximately 2.5. Exhaustive extraction of this filtrate with ether removed only one-fourth of its reacting substances. These ether-soluble phenols showed ratios slightly greater than 1. The ether-insoluble material is a mixture which can be fractionated by lead subacetate. The fraction precipitated by basic lead (approximately two-thirds of the reactive substances) gave high tungstomolybdate to *p*-nitroaniline ratios, while that remaining in the lead filtrate gave low ratios. The lead subacetate precipitate showed no further fractionation by precipitation with barium hydroxide.

The fractional distribution of the ether-insoluble phenols of Lloyd's filtrates is followed quantitatively by the sugar of normal urine. Alkaline mercury filtrates of urine likewise contained



comparable quantities of sugar and phenol, both free and combined. Phenol and sugar reagents are thus reduced by the same urinary fractions and probably by the same constituents.

**Blood Lactic Acid in Rest and Work at High Altitudes.** By H. T. EDWARDS. *From the Fatigue Laboratory, Morgan Hall, Harvard University, Boston*

Resting lactic acid values determined on blood drawn in the morning before rising show an initial slight rise at high altitudes over sea-level values. Sea-level values are found after acclimatization even at 6.14 km., where arterial saturations range between 55 and 70 per cent.

Standard work performances of the subject, on first going to high altitudes, produce greater rises in blood lactic acid than at sea-level. After acclimatization lactic acid values similar to those at sea-level are found for a given performance. The ability to perform work is lessened progressively with increase in altitude, hence, also the ability to accumulate lactic acid. Only slight increases over rest values of lactic acid are found during work at 6.14 km.

The inability to accumulate large amounts of lactic acid at high altitudes suggests a protective mechanism preventing an already low arterial saturation from becoming markedly lower by shift of the  $O_2$  dissociation curve through acid effect. It may be that the protective mechanism lies in an inadequate oxygen supply to essential muscles; *e.g.*, the diaphragm or the heart.

**The Measurement of Blood Cell Volume.** By ANNA J. EISENMAN. *From the Department of Internal Medicine of Yale University School of Medicine, New Haven*

It is possible to determine red cell volume accurately by means of the Daland hematocrit tubes if sufficient care is exercised in the selection, calibration, and cleaning of the tubes and in the manner and speed of centrifugation.

Fourteen experiments of three types were performed to test the accuracy of the method. To whole blood were added dry salt, causing cell shrinkage; water, causing swelling of the cells; or isotonic sucrose solution, which leaves unaltered the absolute volume of the cells, while it causes their relative volume to decrease because of the dilution of the blood.

In each type of experiment, a sample of the same blood specimen was used as control. Hematocrit and serum protein determinations were made in every case. In each of the fourteen experiments, the ratio of serum volumes was found to agree inversely with the ratio of serum proteins. This proves the relative accuracy of the method.

Three experiments where isotonic sucrose solution was added to blood also prove the absolute accuracy of the method. The original cell volume remains unaltered, while the solution was found to expand the serum volume by an amount equal to the amount of solution added. A further check was obtained from the serum solids, as determined by dry weight. Solids in serum of treated blood after proper correction equaled the solids of original serum.

**An Essential Dietary Factor Found in Yeast and Liver Extract Distinct from Vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>4</sub>, and Flavins.** BY C. A. ELVEHJEM, C. J. KOEHN, JR., AND J. J. OLESON. *From the Department of Agricultural Chemistry, University of Wisconsin, Madison*

Young rats placed on a synthetic diet consisting of highly purified casein, dextrin or sucrose, butter or cottonseed oil, salts,<sup>9</sup> cod liver oil, and vitamin B<sub>1</sub> concentrate (Ohdake) fail to grow and die in 4 to 8 weeks without showing any gross symptoms. The addition of a vitamin B<sub>2</sub> concentrate alone, a flavin preparation alone, or a combination of the two, prepared from liver extract and fed in amounts equivalent to 5 per cent of the original liver extract, produced no improvement in growth. However, the addition of 5 per cent of the original liver extract produced a phenomenal growth response. When the basal ration was supplemented with 12 per cent white corn, which supplied sufficient vitamin B<sub>4</sub> to prevent a deficiency of this factor, 2 per cent liver extract or 4 per cent of bakers' yeast gave an equal growth response. The entire activity of the liver extract was found to be present in the alcohol-ether precipitate which is removed in the first part of our method<sup>10</sup> for the purification of vitamin B<sub>2</sub>. The activity of the alcohol-ether precipitate is destroyed by auto-

<sup>9</sup> Keenan, J. A., Kline, O. L., Elvehjem, C. A., Hart, E. B., and Halpin, J. G., *J. Biol. Chem.*, **103**, 671 (1933).

<sup>10</sup> Elvehjem, C. A., and Koehn, C. J., Jr., *J. Biol. Chem.*, **108**, 709 (1935).

claving. Methods for the concentration of this factor are described. The relation of this factor to the known components of the vitamin B complex is discussed.

**Biochemical Studies of Erythrocytes. I. Preparation of a Post-hemolytic Residue.** BY BETTY NIMS ERICKSON, ROBERT L. JONES, SAMUEL S. BERNSTEIN, HAROLD H. WILLIAMS, PEARL LEE, AND ICIE G. MACY. *From the Research Laboratory of the Children's Fund of Michigan, Detroit*

As a part of a study on the chemistry of the erythrocyte efforts have been made to secure for study the cell shadows or "ghosts" which remain after hemolysis of the cells, as observed in the dark-field microscope. Whether this residue consists of the cell membrane or an internal network or "stroma" is a debated question.

Large volumes of washed erythrocytes from different species were hemolyzed by various procedures; namely, by addition of ether, followed by dilution with physiological saline; by addition of water followed by toluene; addition of water followed by addition of 0.1 N HCl to pH 5.5; by freezing and thawing followed by dilution with water; by dilution with 10 to 20 volumes of water buffered with citrate to pH 5.5.

The posthemolytic residues were removed by centrifugation at 35 to 40 thousand R.P.M. in a Sharples supercentrifuge, washed repeatedly until wash solutions gave no evidence of hemoglobin, and dried *in vacuo*. The yield of residue obtained varied from 0.05 to 1 gm. per 100 cc. of erythrocytes. By using total and inorganic iron determinations as criteria for purity of the residue, there was a hemoglobin contamination varying from 64 to 9.5 per cent, depending upon the method of preparation.

A residue has been prepared from hemolyzed erythrocytes that is comparatively free from hemoglobin, the chemical nature of which is being investigated.

**Biochemical Studies of Erythrocytes. II. Lipid Partition of Post-hemolytic Residue.** BY BETTY NIMS ERICKSON, HAROLD H. WILLIAMS, SAMUEL S. BERNSTEIN, AND ROBERT L. JONES. *From the Research Laboratory of the Children's Fund of Michigan, Detroit*

Information concerning the chemical nature of the posthemolytic residue of the erythrocyte should be valuable in throwing light upon its cellular structure. Since lipids are considered of equal importance with proteins in cellular structure, and since such information would furnish an additional index of the purity and uniformity of the various preparations outlined in the previous paper, lipid analyses were made according to the procedure of Kirk, Page, and Van Slyke for blood lipids.

Obviously methods with organic solvents would profoundly disturb the distribution of lipids, and the results obtained bear this out, indicating that residues prepared by less drastic procedures, such as physicochemical means, are more truly representative of the cellular structure.

Results indicate a lipid distribution in these residues similar to that of the intact red cell, such as a relative absence of cholesterol esters and neutral fat and a 2:1 ratio of phospholipid to free cholesterol.

Total nitrogen determinations are in progress and further work will involve a detailed amino acid partition and physicochemical studies of the residue.

**Isomers of Cholesterol.** BY E. A. EVANS, JR. *From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

Isomers of cholesterol may be of considerable biological interest. In cholesterol the double bond is in position (5-6); in allocholesterol it is in position (4-5). By reducing cholestenone with aluminum isopropylate we have obtained a molecular compound of two isomeric unsaturated sterols, of which one is precipitable by digitonin. This precipitable substance differs from the allocholesterol described in the literature in the following respects: it has a higher melting point (130° as compared with 117°), a more positive optical rotation (+43.6° as compared with 0 to +8°), and gives a stronger Rosenheim reaction with trichloroacetic acid. On treatment with dilute HCl it is quantitatively dehydrated to a doubly unsaturated hydrocarbon  $C_{27}H_{44}$  isomeric with cholesterolene; the same hydrocarbon is also formed from the allocholesterol of the literature, together with cholesterol. It

appears from this that the compound melting at  $130^{\circ}$  is pure allocholesterol, while the product described in the literature is a mixture of allocholesterol and cholesterol.

The properties of the non-precipitable sterol (epiallocholesterol) have recently been described by us.<sup>11</sup> This substance also quantitatively yields the hydrocarbon  $C_{27}H_{44}$  on treatment with dilute HCl. The ease with which this hydrocarbon is formed by these sterols is characteristic of the lability of hydroxyl groups adjacent to a double bond.

**The Solvent Action of Neutral Salts upon Carboxyhemoglobin in Solutions of Low Dielectric Constant.** BY RONALD M. FERRY, EDWIN J. COHN, AND ETHEL S. NEWMAN. *From the Department of Physical Chemistry, Harvard Medical School, Boston*

The solvent action of neutral salts upon amino acids and peptides in media of low dielectric constant has previously been shown by Cohn, McMeekin, and Greenstein to depend, as a first approximation, upon the ionic strength of the salt and the di-pole moment of the zwitter ion.

The solubility of horse carboxyhemoglobin, a protein previously investigated in aqueous salt solutions by Cohn and Prentiss, and by Green, has now been studied in 25 per cent ethanol at  $-5^{\circ}$ , with the methods employed by us in the study of egg albumin. The carboxyhemoglobin was first recrystallized from aqueous solution, then from 25 per cent ethanol at low temperatures. The crystals were repeatedly washed with the solvent before equilibration. The solvent action of sodium chloride upon carboxyhemoglobin in 25 per cent ethanol is but slightly greater than in water, and far less than upon egg albumin in 25 per cent ethanol.

Although the radius of hemoglobin is nearly 1.5 times that of egg albumin, and although it contains over 3 times as many ionizable di-poles, these results suggest that their spatial arrangement is such as to yield, as a vector sum, a smaller electric moment. Other solubility studies from this laboratory suggest that this hypothesis is consistent with the facts that horse carboxyhemoglobin is much less soluble in water, and more readily precipitable by neutral salts, than egg albumin and with its consequent classification as a globulin.

<sup>11</sup> Evans, E. A., Jr., and Schoenheimer, R., *J. Am. Chem. Soc.*, **58**, 182 (1936).

Other proteins, among them human hemoglobin, whose behavior in concentrated salt solutions suggests a more albumin-like molecule, are being similarly investigated.

**Chemical Adjustments to High Temperature.** BY ELLA H. FISHBERG, WILLIAM BIERMAN, AND ALTER WEISS. *From the Chemical Laboratory of the Beth Israel Hospital, New York*

The therapeutic administration of fever by means of short wave to a large number of patients suffering from varied ailments offered a unique opportunity for the study of the chemical changes in the body in response to high temperature, uncomplicated by extraneous factors. The phenomena to be reported affected practically every constituent of the blood, tissue fluids, and urine. A few of these changes may be summarized. The hyperventilation brings about an extreme lowering of the carbon dioxide in the blood and alveolar air. The  $\text{CO}_2$  dissociation curve is lowered. The pH is raised to 7.6. The fixed base is lowered. Lactic acid is high in the blood and enormous quantities are lost through the sweat. The lowering of  $\text{CO}_2$  pressure in the alveolar air and in the body as a whole results in a firmer attachment of the oxygen to the hemoglobin, with consequent oxygen-want in the tissues. Hemoglobin is greatly increased. The blood inorganic phosphorus is doubled.

The pH of the urine reaches 8, with a very high carbonate content, chlorine being practically absent. Free hydrochloric acid disappears from the gastric juice.

Breathing is extremely irregular, the respiration becoming shallow and rapid. This tachypnea alternates with short periods of true hyperpnea which are followed by periods of apapnia. The long apneic periods are due to the washing out of the carbon dioxide plus the spastic condition of the musculature. A Trousseau's sign may be elicited.

**Variations in Blood Cholesterol Following the Intravenous Administration of Cholesterol.** BY FRED FITZ AND MAURICE BRUGER. *From the Department of Medicine, New York Post-Graduate Medical School and Hospital, New York*

Recent balance experiments have demonstrated the ability of the animal organism to destroy cholesterol administered by

mouth; the present study was designed to determine the fate of cholesterol given intravenously. A colloidal suspension of free cholesterol was made with certain modifications after the manner described by Dewey. Amounts varying from 960 to 1600 mg. of cholesterol, calculated to increase the blood cholesterol approximately 50 per cent, were injected intravenously into non-anesthetized dogs during approximately 10 minutes. Blood specimens were obtained immediately before and at intervals of 10, 20, 40 minutes, and 1, 2, 4, 6, and 24 hours after the injection.

In about one-half of the experiments no increase in blood cholesterol was observed over a period of 6 hours following the intravenous injection. In the remainder of the experiments there occurred a transient hypercholesterolemia that persisted for less than 1 hour. Most of the animals showed a moderate rise in the blood cholesterol approximately 24 hours later. In several dogs with extensive hepatic damage produced by chloroform as evidenced by marked dye retention, no greater augmentation of the hypercholesterolemia was observed following the intravenous administration of cholesterol than occurred in normal animals.

This preliminary study is being extended at present by subjecting rabbits to the same type of experiment; tissue analyses for cholesterol are also being carried out. It is hoped that this will aid in determining whether intravenously injected cholesterol is deposited in the tissues, is rapidly destroyed, or is excreted.

**The Effect of Diet upon the Phosphate Compounds in the Liver of the Dog.** BY EUNICE FLOCK, JESSE L. BOLLMAN, AND FRANK C. MANN. *From the Division of Experimental Medicine, The Mayo Foundation, Rochester, Minnesota*

Values for total phosphorus in the liver varied from 240 to 320 mg. per 100 gm. of tissue when the animals were allowed free access to a mixed diet. When weighed amounts of diets of similar nature were used, the spread of values was narrower in a group of selected dogs. The total phosphorus was divided roughly into thirds, the acid-soluble phosphates, the phospholipids, and the residual phosphates. The acid-soluble phosphates included the inorganic, a labile nucleotide, glycerophosphate, and compound X, of high solubility. The labile nucleotide was broken down in 15 minutes by autolysis or acid hydrolysis.

Of a variety of diets used the only one which produced definitely abnormal phosphate values was the high fat diet. Following the administration of this for 3 weeks or longer the liver glycogen was extremely low, the water content markedly reduced, and the fatty acids increased to very high levels. In these fatty livers all of the phosphate values were abnormally low. Of the three main divisions the biggest decrease from normal occurred in the acid-soluble phosphates. In the acid-soluble group, all of the organic phosphates showed a greater decrease than the inorganic phosphates. A replacement of this high fat diet by a carbohydrate diet produced a rapid restoration of normal values.

**The Endogenous Nitrogen Excretion in Relation to the Determination of Biological Value of Protein.** BY R. B. FRENCH AND H. A. MATTILL. *From the Biochemical Laboratory, State University of Iowa, Iowa City*

This paper discusses the nitrogen balances of 103 protein-free feeding trials made on rats in connection with determinations of biological value. Studies of the length of an experimental period necessary to secure a constancy of nitrogen excretion showed that young, adolescent, and mature animals did not excrete nitrogen in a comparable manner. The age of the animal as well as its previous feeding history greatly influenced the rate of nitrogen excretion in a subsequent protein-free feeding period.

Endogenous fecal nitrogen excretion correlated fairly well with body weight but not with the food intake. If a part of the fecal metabolic nitrogen results from ingestion of food it is suggested that, in the computation of biological values, this part should be apportioned to the cost of utilization and not of maintenance.

**The Direct Determination of Ethyl Alcohol in Saliva without Distillation.** BY THEODORE E. FRIEDEMANN AND THEODORE BROOK. *From the Laboratory of Chemical Bacteriology, Department of Medicine of the University of Chicago, Chicago*

1 cc. of saliva and 10 cc. of a solution consisting of 4 per cent  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 4 per cent  $\text{HgSO}_4$ , and 1.5 per cent  $\text{Fe}_2(\text{SO}_4)_3$  are measured into a 100 cc. volumetric flask. 50 cc. of  $\text{H}_2\text{O}$  and an excess of a suspension of  $\text{Ca}(\text{OH})_2$  are then added. After mixing, the volume is brought to the mark. 10 cc. of the supernatant



solution are measured into a flask. To this are added 15 cc. of  $\text{H}_2\text{O}$ , 10 cc. of 5 N NaOH, and 25 cc. of 0.01 N  $\text{KMnO}_4$ . The contents are thoroughly mixed and then heated in a boiling water bath. After cooling, the contents of the flask are acidified, and the residual  $\text{KMnO}_4$  is determined iodometrically. The quantity of alcohol oxidized is measured by the difference between the titration of the sample and the titration of a blank. 1 cc. of 0.01 N  $\text{KMnO}_4$  is equivalent to 0.0420 mg. of ethyl alcohol. The results obtained by direct oxidation, when compared with those obtained by distillation, differ by a constant value,  $12 \pm 5$  mg. per 100 cc.

**Precipitation of Apparent Creatinine from Ultrafiltrates of Normal Sera.** BY OLIVER HENRY GAEBLER. *From the Department of Laboratories, Henry Ford Hospital, Detroit*

The writer has previously reported a procedure by which most of the apparent creatinine in cellophane ultrafiltrates of nephritic sera can be precipitated directly by addition of picric acid and potassium chloride. By substituting rubidium chloride for potassium chloride in this procedure, and carrying out the precipitation in an ordinary refrigerator, it has become possible to precipitate most of the apparent creatinine from cellophane ultrafiltrates of normal sera. In a series of dog sera obtained during the postabsorptive period the apparent creatinine of the ultrafiltrates ranged from 1.22 to 1.65 mg. per 100 cc., and of this only 0.50 to 0.62 mg. remained unprecipitated after 48 to 72 hours in the refrigerator. The use of rubidium was based upon the experiments of Greenwald and Gross with known creatinine. In 1924 they reported that rubidium creatinine picrate is less soluble than the corresponding potassium compound, and suggested its use in connection with isolation of blood creatinine.

Ultrafiltrates prepared on a large scale, with collodion membranes and apparatus similar to that of Wilson and Holiday, can also be used in this procedure, although the precipitation in this case is somewhat slower with normal serum ultrafiltrates. An ultrafiltrate prepared in this way from human serum showing marked retention yielded a yellow granular precipitate after addition of picric acid and before any rubidium chloride was added. This precipitate gave no Jaffe reaction, and on decom-

position with ether and hydrochloric acid yielded a crystalline residue of which the water-insoluble portion was uric acid.

**Effects of an Anterior Pituitary Growth Preparation on Sulfur Metabolism.** BY OLIVER HENRY GAEBLER AND W. H. PRICE.  
*From the Department of Laboratories, Henry Ford Hospital, Detroit*

Dogs on a constant diet were given single large doses of a growth preparation from beef anterior lobes (antuitrin G). The nitrogen and phosphorus balances and the inorganic, ethereal, and total sulfur of the urine were determined.

The fall in total sulfur of the urine after the injection is even more striking than the fall in nitrogen excretion which was previously studied under similar conditions. The N:S ratio of the urine increases. The decrease in total sulfur is primarily due to a fall in inorganic sulfate excretion, although neutral sulfur output also diminishes. During the 2nd and 3rd weeks after injection, when nitrogen is lost again, sulfur excretion also rises above the control level.

Since our diets contained bone meal, the phosphorus content of the feces was large, and an effect of the injection on the phosphorus balance was not demonstrated. The phosphate content of the urine, however, ran parallel with the nitrogen and sulfur content, falling to an extremely low level after the injection and exceeding the control level during the subsequent period of nitrogen loss.

The results are consistent with the idea that the retained nitrogen is converted into protein. No lactation occurred in the current experiments, although the growth preparation was not free of the lactation factor.

**The Comb Growth Reaction to Synthetic Male Hormone Preparations.** BY T. F. GALLAGHER AND F. C. KOCH. *From the Department of Biochemistry of the University of Chicago, Chicago*

Comparative studies on androsterone, androstenedione, androstenediol, dehydroandrosterone, and testis extracts show the different absolute activities but the same relationship of dose to comb growth. The results show that the comb growth method is adequate for the biological assay of these products.

**The Immunological Properties of Artificial Antigens Containing Glucose and Glucuronic Acid.** BY WALTHER F. GOEBEL AND KENNETH GOODNER. *From the Hospital of The Rockefeller Institute for Medical Research, New York*

Artificial carbohydrate-protein antigens, prepared by combining the diazonium derivatives of the *p*-aminobenzyl  $\beta$ -glycosides of glucose and glucuronic acid with foreign protein, give rise in rabbits to antibodies which are immunologically distinct and specific. It has been found that the glucuronic acid-protein antigen reacts in dilutions of 1 part in 1 million with antipneumococcus horse sera, Types II, III, and VIII, whereas the corresponding glucose antigen is serologically inactive. Thus the artificial glucuronic acid-protein complex bears a striking immunological relationship to the naturally occurring pneumococcus specific polysaccharides. The chemical basis for this immunological similarity probably resides in the uronic acid component, for it has been shown that the specific polysaccharides of Types III and VIII pneumococcus (and probably Type II) contain glucuronic acid as a part of the complex polysaccharide molecule. The serological activity of the artificial glucuronic acid-protein antigen in Types II, III, and VIII antipneumococcus sera may be attributed to the interaction of uronic acid antibodies, elicited by the highly polar uronic acid constituent of the bacterial polysaccharide, with the uronic acid radical of the artificial antigen.

Although antipneumococcus immunity may of course be readily induced by immunization with vaccines of Types II, III, or VIII pneumococci, it has as yet not been possible to produce antipneumococcus immunity, either in rabbits or in mice, with the artificial glucuronic acid-protein antigen. The efficaciousness of this artificial antigen in other animal species is at present being investigated.

**The Protective Action of Non-Saponifiable Matter of Soy Bean Oil in Nutritional Encephalomalacia of Chicks.** BY MARIANNE GOETTSCH AND ALWIN M. PAPPENHEIMER. *From the Departments of Biological Chemistry and Pathology, College of Physicians and Surgeons, Columbia University, New York*

It has been shown that a synthetic diet containing all the factors

known to be necessary for normal rat growth produces in young chicks a severe disorder of the brain, for which the term nutritional encephalomalacia has been proposed. Chicks were found to be protected from this disease by the addition to the diet of various vegetable oils.

Soy bean oil was chosen for fractionation studies because it appeared to be richer in the protective factor than the other oils studied.

The activity of the oil was not changed by autoclaving for 6 hours at 120°, nor by aeration for 48 hours.

A 20-fold concentration of the active material was effected by extraction of the oil with 95 per cent alcohol at room temperature. This alcoholic extract retained its activity after 1 year's storage in the refrigerator.

The activity was completely destroyed by saponification in the presence of air, but not affected by saponification in an atmosphere of nitrogen. The non-saponifiable fraction protected chicks, but neither the fatty acid fraction nor the water-soluble portion had any activity. For separation of the non-saponifiable matter it was found necessary to remove the impurities present in petroleum ether with concentrated sulfuric acid.

Preliminary experiments indicate that the addition of soy bean oil to the diet prevents nutritional muscle dystrophy of rabbits and guinea pigs and nutritional myopathy of ducklings.

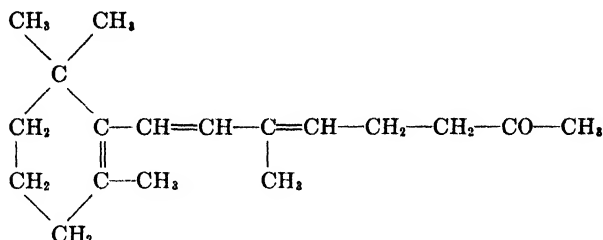
**The Synthesis of a Dihydrovitamin A.** BY R. G. GOULD, JR.\*

*From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

As a contribution to the question of the relationship of chemical structure to vitamin A activity,  $\gamma$ ,  $\delta$ -dihydrovitamin A has been synthesized from  $\beta$ -ionone.

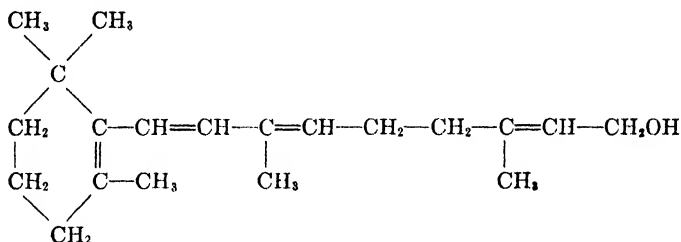
\* National Research Council Fellow in Chemistry.

By the method given by Gould and Thompson<sup>12</sup> the ketone



was prepared from  $\beta$ -ionone.

This ketone was condensed with acetylene, 1 mole of hydrogen was added to give the vinylcarbinol, and this was rearranged by treatment with acetic anhydride to give the isomeric  $\alpha, \beta$  unsaturated primary alcohol.



Purification of this compound through the phthalic acid ester proved it to be a primary alcohol, and its structure was further established by analysis and by the presence of an absorption band at 281  $m\mu$ .

Preliminary tests for vitamin A activity indicate that this compound is completely inactive.

**The Blood Coagulant Derived from the Placenta.** BY ARDA ALDEN GREEN, HOPE LOWRY, R. C. ELEY, AND CHARLES F. MCKHANN. *From the Department of Pediatrics, Harvard Medical School, Boston*

The blood coagulant derived from the human placenta is a large protein molecule of tissue globulin precipitated in isotonic

<sup>12</sup> Gould, R. G., Jr., and Thompson, A. F., Jr., *J. Am. Chem. Soc.*, **57**, 340 (1935).

saline at pH 5. It decreases the coagulation time of normal and hemophiliac blood. It has a certain degree of species specificity, forms part of the fibrin clot, and catalyzes the formation of thrombin from prothrombin.

**Pathological Changes in the Tissues of Rats Reared on Diets Low in Magnesium.** BY DAVID M. GREENBERG, CARL E. ANDERSON, AND ELMA V. TUFTS. *From the Division of Biochemistry, University of California Medical School, Berkeley*

Histological examination of the tissues of rats reared on a diet deficient in magnesium (1 to 2 mg. of Mg per 100 gm. of dry food) revealed evidence of marked degenerative changes, particularly in the kidneys, but to some extent also in the heart. The pathological changes in the heart appear to consist of a myocardial degeneration with fibrosis and polyblastic infiltration. The histological picture in the kidney consists of multiple casts which are stained purple with hematoxylin, found most prominently in the medullary loops and the collecting tubules. These areas also show wide-spread tubular degeneration with fibrosis and polyblastic infiltration. The renal cortex shows tubular and glomerular degeneration with demarcated, periglomerular zones of fibrosis and polyblastic infiltration. Besides histological changes, the kidneys also show such gross changes as light colored patches or pitting of the kidney surface and irregular coloring in the cortex of kidney slices.

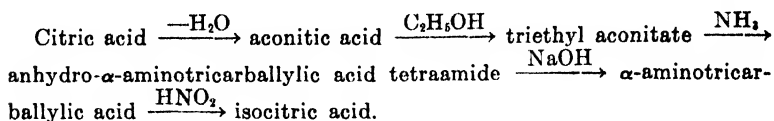
These changes were noted in animals which had been on the deficient diet anywhere from 18 to 120 days, the degree of degeneration increasing with the time on the diet. On the other hand, none of the control animals, which, with the exception of receiving 50 mg. of Mg per 100 gm. of food, were on the same diet, showed any evidence of gross or histological changes in the kidneys or heart.

**The Synthesis of Isocitric Acid from Citric Acid.** BY JESSE P. GREENSTEIN. *From the Department of Physical Chemistry, Harvard Medical School, Boston*

Isocitric acid ( $\alpha$ -hydroxytricarballic acid) was first synthesized by Fittig by the decomposition of trichloromethylparaconic acid

with baryta. It was isolated in the optically active form from blackberries by E. K. Nelson in 1925.

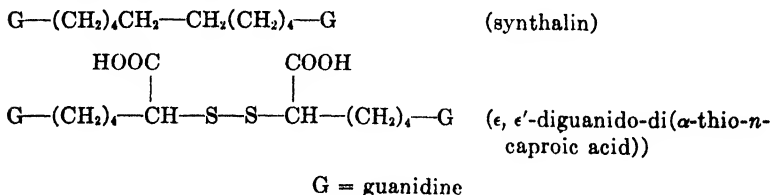
The acid has been synthesized in this laboratory from citric acid ( $\beta$ -hydroxytricarballic acid) by the following series of reactions:



The isocitric acid was identified by means of its barium salt and by the crystalline calcium salt of the corresponding lactone. The yield was extremely low; from 2.5 kilos of citric acid only 25 gm. of isocitric acid were obtained. The loss is to be largely traced to formation of the diketopiperazine, the yield at this step sinking to 14 per cent of the theory.

**The Effect of Certain Substituted Dithio Acids on the Blood Sugar of Rabbits.** BY JESSE P. GREENSTEIN AND HARRY B. FRIEDGOOD. *From the Department of Physical Chemistry and the Department of Physiology, Harvard Medical School, Boston*

Synthalin administration produces liver and kidney damage, transitory hyperglycemia, and subsequent severe hypoglycemia in doses of 10 to 20 mg. per kilo. In an attempt to obviate these toxic effects and retain this hypoglycemic activity, certain chemical characteristics of the insulin molecule (disulfide linkage; guanidine, amino, carboxyl and phenyl groups) were incorporated in the synthesis of three acids also containing the molecular configuration of synthalin. These acids were  $\epsilon, \epsilon'$ -diguano-di( $\alpha$ -thio-*n*-caproic acid),  $\epsilon, \epsilon'$ -diamino-di( $\alpha$ -thio-*n*-caproic acid), and  $\epsilon, \epsilon'$ -diphenylureido( $\alpha$ -thio-*n*-caproic acid).



This diguanido acid was physiologically inactive when administered intravenously in doses of 20 to 50 mg. per kilo. Four of

the five rabbits receiving 100 to 175 mg. per kilo showed transient hyperglycemia (one had mild transitory tremors). Three rabbits given 200 to 250 mg. per kilo developed hyperglycemia, dyspnea, and severe convulsions; two of these died rapidly. The blood sugar level increased within the 1st hour and became normal by 5 to 6 hours in all surviving animals which had had hyperglycemia. Histological examination of liver and kidney was negative.  $\epsilon, \epsilon'$ -Diamino-di( $\alpha$ -thio-*n*-caproic acid) had no observable physiological effect in an intravenous dose of 245 mg. per kilo;  $\epsilon, \epsilon'$ -diphenylureido-di( $\alpha$ -thio-*n*-caproic acid) was similarly ineffective orally or subcutaneously (suspended in water) in doses of 10 to 20 mg. per kilo. The latter preparation, being highly insoluble, was difficult to administer.

The introduction of two  $\alpha$ -carboxyl groups and of a disulfide linkage in the center of the synthalin molecule radically alters its toxicity and physiological activity. The hyperglycemic effects of the diguanido acid were probably elicited through stimulation of the nervous system.

**The State of Calcium in Serum and in Plasma.** BY ISIDOR GREENWALD AND SOLOMON H. RUBIN. *From the Department of Chemistry, New York University College of Medicine, New York*

In the series of papers by McLean and Hastings and their co-workers, no consideration is given to the fact that Miyamoto and Schmidt found that casein bound approximately twice as much calcium as sodium at the same pH, nor to the possible existence of calcium-carbamino-protein. In either case, the equation is not

$$\frac{(\text{Ca}^{++})(\text{Prot}^-)}{(\text{CaProt})} = K$$

but

$$\frac{(\text{Ca}^{++})(\text{total Prot}^- - \text{CaProt})}{(\text{CaProt})} = K$$

in which  $\text{CaProt}$  = Ca bound non-ionically to protein. The latter equation is more in accord with the observations than is the former.

Calcium-carbamino-protein compounds probably occur, but



the fact that protein binds calcium in the absence of  $\text{CO}_2$  and, also, calculations from the results of Dillman and Visscher indicate that other groups may be involved.

Analyses of  $\text{BaSO}_4$  prepared by the method of Benjamin and Hess showed it to contain  $\text{Ba}(\text{NO}_3)_2$ , whereas  $\text{BaSO}_4$ , No. 4, prepared according to the directions of Greenberg and Larson, contained  $\text{Na}_2\text{SO}_4$ . By ion interchange, the former absorbs  $\text{PO}_4^{--}$  from solutions not containing calcium and the latter  $\text{Ca}^{++}$  from solutions not containing phosphate.

The authors believe that serum is supersaturated with respect to calcium-carbonato-phosphate. However, from a consideration of the phosphorus excretion after parathyroidectomy, it is concluded that a non-ionic, but diffusible, form of calcium, if present, would disappear in less than 12 hours and, in some cases, in 30 minutes after the operation. The previous claim to its demonstration in serum is withdrawn.

**A Further Quantitative Study of the Estrogenic Substances in Normal Human Female Urine.** BY REUBEN G. GUSTAVSON, TOM WOOD, AND ERWIN HAYS. *From the Chemical Laboratories of the University of Denver, Denver*

24 hour samples of normal female urine have been extracted with chloroform for 24 hours. 60 liters of chloroform were circulated against the urine during this period. The extract was partially purified and then emulsified with water, with glycol stearate as the emulsifying agent. The extract was assayed by use of spayed rats by the vaginal smear method. The material was assayed at two dilutions. Ten animals were used for the higher concentration and fifteen animals for the lower concentration. The dosage was given in four injections 12 hours apart.

There is a marked rise in estrogenic substances about 11 days after menstruation, followed by a rapid decline for 2 or 3 days. This is followed by a second rise which falls off gradually. Attempts have been made to determine the estrogenic substances colorimetrically by the method of Kober, as modified by Cohen and Marrian. No correlation has been found between the colorimetric assay and the biologic assay. This confirms the findings of Marrian.

In order to express the results in terms of international rat

units, the international standard of "estrus-producing hormone" has been carefully assayed. With a single injection of the material dissolved in oil it was found that 1.3 micrograms were necessary to bring 50 per cent of 50 rats into estrus. With multiple injections 12 hours apart and with the material in aqueous suspension it was found that 0.76 microgram was necessary to bring 50 per cent of the 50 rats into estrus.

### **The Acid-Base Changes in the Blood after Anaerobic Work.**

BY A. BAIRD HASTINGS, D. B. DILL, AND H. T. EDWARDS.  
*From the Fatigue Laboratory, Morgan Hall, Harvard University,  
and the Department of Biological Chemistry, Harvard Medical  
School, Boston*

An attempt has been made to study in a trained athlete the significant acid-base changes in the blood, during the recovery period following maximum work of an intensive nature. Samples of blood from the femoral artery, femoral vein, and antecubital vein were obtained within the first half minute, and subsequently at frequent intervals up to 1 hour.

From observations of the pH, CO<sub>2</sub>, O<sub>2</sub>, and lactic acid of the blood, it was found that: (1) Immediately after the exercise a great excess of CO<sub>2</sub> existed in the tissues of the leg. This excess was rapidly eliminated within the first 6 minutes of the recovery period. (2) Although the lactic acid of the bloods from all three sources was increased greatly above normal immediately after exercise, it continued to increase in the femoral, venous, and arterial blood for approximately 6 minutes before beginning to decrease. The lactic acid of the blood from the antecubital vein continued to increase for an even longer period. (3) Removal of lactic acid in the passage of blood through the arm was observed.

### **The Phospholipid Metabolism of Tumors.** BY FRANCES L.

HAVEN. *From the Department of Biochemistry and Pharmacology, The University of Rochester School of Medicine and Dentistry, Rochester, New York*

Phospholipids of the normal cell have been assigned various functions; namely, those of fat metabolism, of oxygen transport, and of cellular structure. Recently, Sinclair has shown that at least two classes of phospholipid may exist, one functioning as an

intermediate in fat metabolism and the other in cellular structure. Liver has been shown by him to contain both classes of phospholipid, while muscle contains mainly non-metabolic phospholipid. This conclusion was reached by feeding elaidin and showing that elaidic acid replaces part of the normal phospholipid fatty acids of the rat, that its entrance into and disappearance from the phospholipids is rapid in liver but comparatively slow in muscle.

The phospholipid content of tumors is high, but the exact function of phospholipids here as in normal cells is unknown. The functions mentioned above for normal cells might explain the permeability, the respiration, or the low respiratory quotients of tumors.

By including elaidin in the diet of rats inoculated with carcinosarcoma No. 256, it has been found that elaidic acid replaces some of the normal fatty acids in tumor phospholipids. Moreover, the entrance of elaidic acid into and disappearance from tumor phospholipids is comparatively slow. Therefore, the phospholipids of tumor, unlike those of liver, have little, if any, metabolic function. Instead, they resemble those of skeletal muscle in being mainly of the non-metabolic type.

**The Determination of Vitamins B<sub>1</sub> and B<sub>2</sub> in Human Urine.** By O. M. HELMER. *From the Lilly Laboratories for Clinical Research, Indianapolis City Hospital, Indianapolis*

The 24 hour urine samples from normal subjects who ate a weighed amount of a well balanced diet were adjusted to pH 4.0 with hydrochloric acid and shaken with Lloyd's reagent. The Lloyd's reagent was collected by means of vacuum filtration on a hardened filter paper, washed with water, and dried *in vacuo*. The Lloyd's adsorbate in amounts equivalent to one-fiftieth of the daily urine output was fed to rats on a vitamin B complex-free basal diet as a sole source of the B complex, and also with the addition of a vitamin B<sub>1</sub> or B<sub>2</sub> preparation. The growth rate of the rats indicated that appreciable amounts of vitamins B<sub>1</sub> and B<sub>2</sub> are excreted in the urine and that the Lloyd's reagent quantitatively adsorbed these vitamins.

**The Effect of Denaturation of Egg Albumin upon Its Acetyl Derivatives.** BY BYRON M. HENDRIX AND FELIX PAQUIN, JR.

*From the Laboratory of Biological Chemistry, School of Medicine, University of Texas, Galveston*

Acetyl egg albumins prepared from crystallized egg albumin, from alcohol-coagulated egg albumin, and from heat-coagulated egg albumin, are the same in so far as the acetyl content is concerned. The amount of acetyl per gm. of nitrogen is equivalent to an average of 119 cc. of 0.1 N acetic acid.

Acetyl egg albumin prepared from alkali-treated egg albumin contains much less acetyl. One specimen contains the equivalent of 42 cc. of 0.1 N acetic acid. Other samples contain even less. Acetyl egg albumin prepared from acid-treated egg albumin contains only 27.15 cc. of 0.1 N acetyl per gm. of nitrogen.

This effect of treatment with acid or alkali can be demonstrated on other proteins; for example, acetyl casein prepared from acid- or alkali-treated casein contains much less acetyl than the acetyl casein which has been prepared from untreated casein. Edestin gives similar results.

These results may indicate that coagulation does not affect the acetylation of protein, but it is more probable that the crystallized egg albumin is coagulated by the acetic anhydride during the acetylation.

It is certain, however, that proteins which have been previously dissolved in dilute acid or alkali and reprecipitated are so changed that they combine with less acetyl than the untreated protein.

These facts support the idea that there is a fundamental difference between the coagulation by heat or alcohol at the isoelectric point and the denaturation of protein by the action of acid or alkali.

**The Cystine Content of Deaminized Proteins.** BY W. C. HESS AND M. X. SULLIVAN. *From the Chemo-Medical Research Institute, Georgetown University, Washington*

Casein, lactalbumin, and wool were deaminized according to the method of Dunn and Lewis.<sup>13</sup> The cystine content of these deaminized proteins was determined by the Sullivan, Okuda,

<sup>13</sup> Dunn, M. S., and Lewis, H. B., *J. Biol. Chem.*, **49**, 327 (1921).

Folin-Marenzi, Vickery and White, and Shinohara methods. The Sullivan method, in each case, gave cystine values considerably lower than for the pure proteins. The cystine content of the deaminized proteins found by the Okuda method was either the same or slightly lower than that of the untreated proteins. The Folin-Marenzi values were considerably higher for the deaminized proteins than for the undeaminized. The Shinohara values vary somewhat, but on the average are slightly higher for the deaminized protein than for the untreated. The method of Vickery and White gave approximately the same results upon both the untreated and deaminized proteins. Hydrolysis with 8 N  $\text{H}_2\text{SO}_4$  gave higher values in most of the methods than did hydrolysis with 20 per cent HCl. It was found that deaminized cystine prepared from pure cystine was negative in the Sullivan method but gave positive reactions with all the other methods. From the fact that the Sullivan method gave values for the proteins lower than the other methods employed it can be concluded that upon deaminization the cystine is changed more or less, but the sulfur is left relatively unchanged.

**The Action of Alkali upon Dibromoxyhydrouracil.** BY FRANCIS F. HEYROTH. *From the Basic Science Research Laboratory, University of Cincinnati, Cincinnati*

Aqueous or alcoholic solutions of dibromoxyhydrouracil react with sodium hydroxide to yield a series of pyrimidine derivatives. With 1 to 4 moles of alkali per mole of the uracil derivative, red sodium diisobarbiturate precipitates, the maximum yield (20 per cent) being obtained with 2.5 moles. The mechanism proposed for its formation involves the intermediary formation of isobarbituric acid, which is oxidized by simultaneously produced hypobromite. The filtrate contains only small quantities of dialuric acid when more than 1.5 moles of alkali is used. Alkaline filtrates undergo slow atmospheric oxidation (which may be hastened by small amounts of potassium ferrieyanide), depositing very small amounts of a dark unidentified substance, which in aqueous solution exhibits indicator properties. It is yellow at  $\text{pH} < 2$ , blue from  $\text{pH} 2$  to approximately 6, and reddish purple at  $\text{pH} > 6$ . The blue dye is decolorized by sodium hydrosulfite and reoxidized by air.

Other and different indicators are produced by the acid ferricyanide oxidation of dibromoxyhydrouracil or isodialuric acid. The significance of these new pyrimidine indicators as respiratory catalysts is being studied.

**Semiquinones of Anthraquinone Sulfonates.** BY EDGAR S. HILL AND PHILIP A. SHAFFER. *From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis*

The oxidation-reduction systems in which semiquinones have so far been demonstrated show evidence of separation into two 1 valence steps only in the more acid region, with the exception of the indigo sulfonates where separation appears (and disappears) at high pH. With the object of finding relatively simpler systems with two-step oxidation-reduction in alkaline solution the electrochemical behavior of the anthraquinone sulfonates, previously studied by Conant, Kahn, Fieser, and Kurtz, has been resurveyed. The changing slope of the titration curves with pH noted by these workers is due to semiquinone (rather than meriquinone) formation, evidenced by the fact that change of concentration has slight or no effect on the potential curves. The behavior of these substances suggests that many more systems may be capable of two-step oxidation-reduction reactions than are readily demonstrated by their titration curves. A possible significance of this property in catalytic action will be noted.

**Observations on the Anemia Caused by Deaminized Casein.**

BY ALBERT G. HOGAN AND RALPH E. GUERRANT. *From the Department of Agricultural Chemistry, University of Missouri, Columbia*

Gelatin and gliadin together are a source of complete protein, but, when deaminized casein was also included, rats that consumed the combination invariably became anemic and died. When deaminized casein was combined with untreated casein, anemia did not develop and the animals grew normally.

Lactalbumin was ineffective in healing anemia caused by deaminized casein. Commercial dried ovalbumin healed the condition promptly, but results were variable when an attempt was made to purify the preparation. Dried yeast was effective in healing, but only at high levels, approximately 18 per cent.

When the yeast, or casein, was autoclaved the antianemic activity was lost. Casein that had been hydrolyzed with strong acid was likewise ineffective. When the rations contained 10 per cent of deaminized casein, the minimum amount of casein that would prevent anemia lay between 2.5 and 5 per cent of the ration. Under our experimental conditions the minimum amount of deaminized casein that causes anemia lies between 5 and 10 per cent. A number of miscellaneous products were examined for antianemic potency, including milk, egg yolk, water extract of yeast, wheat germ oil, and a number of animal tissues. The amounts supplied ranged from 100 to 400 mg. daily, but in no case was any important curative action observed.

**X-Ray Absorption Coefficients of Dentin and Enamel.** BY FRANKLIN HOLLANDER. *From the Laboratory of Physical Chemistry, Department of Oral Histology, School of Dental and Oral Surgery, Columbia University, New York*

By means of a precision method<sup>14</sup> for measuring linear x-ray absorption coefficients ( $\mu$ ) in 0.25 c.mm. samples, a series of measurements was made on enamel ( $En$ ) and coronal dentin ( $D$ ) of four human teeth. The averages were:  $En = 189 \pm 1.9$  (s. d. for eighteen values) and  $D = 97 \pm 0.8$  (thirty-one values), expressed as micra of aluminum equivalent to 100 micra of tooth substance; the  $En/D$  ratio  $= 1.94 \pm 0.02$ , as against a theoretical value of 1.8. The latter was calculated by an approximate empirical formula<sup>15</sup> involving the cube of the atomic number for each constituent, the weights per cent (derived from a typical analysis by Logan), and specific gravity of the sample. In view of the acknowledged uncertainty of the formula as applied to compounds, and the random nature of the analytical data, the observed and calculated values are in good agreement.

In order to determine the significance of  $\mu$  measurements for studies in dental calcification, a comparison was made of  $En/D$  ratios, calculated from the same analytical data, and expressed in terms of the following criteria of calcification: (1) (mg. of Ca (or P))/(100 mg. of sample), (2) (mg. of Ca)/(c.mm.), and (3)  $\mu$ .

<sup>14</sup> Hollander, F., *Proc. Soc. Exp. Biol. and Med.*, **33**, 388 (1935).

<sup>15</sup> Washburn, E. W., *International critical tables of numerical data, physics, chemistry and technology*, New York, **6**, 12 (1929).

These ratios equal 1.3, 1.8, and 1.8 respectively, and represent the relative degree of calcification of *En* and *D* as measured by these several criteria. Physiological considerations of dental calcification should be based on Ca (or Ca salt) content per unit volume, *i.e.* density of calcification, rather than content per unit weight. Since the *En/D* ratios in terms of (3) and (2) agree, but that based on (1) is different, it follows that  $\mu$  values may serve as a measure of density of calcification, whereas (1) cannot.

**Studies on Digestibility of Proteins in Vitro. VII. Rate of Liberation of Cystine on Tryptic Digestion of Casein, with Observations on the Stability of Cystine in Digestion Mixtures at Different pH Values.** BY D. BREESE JONES AND CHARLES E. F. GERSDORFF. *From the Protein and Nutrition Research Division, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington*

When casein is digested *in vitro* with trypsin at pH 8 to 9, cystine is early liberated, but at the same time is largely destroyed by the alkaline reaction of the digestion mixture.

In order to be able to measure quantitatively the rate of liberation of cystine it was found necessary to conduct the digestion at neutrality, or slightly on the acid side. At pH 6.8, 23 per cent of the total cystine was liberated at the end of 4 hours. It was liberated completely in 135 hours. At pH 6.6 and 6.2, the rate of digestion was slower, 85 per cent being liberated in 144 hours at pH 6.2.

Digestions conducted on the alkaline side revealed some interesting facts relative to the stability of cystine. (1) With tryptic digestion of casein in alkaline medium the destruction of cystine is greater with sodium hydroxide than with sodium carbonate. (2) After a solution of cystine alone was digested for 4 hours at pH 8 to 9, all the cystine was recovered, but when digested under the same conditions in the presence of casein and trypsin only 47 per cent of the cystine added could be recovered. (3) Digestions of casein with and without trypsin, under otherwise the same conditions, for 24, 48, and 72 hours at pH 8 to 9 showed that in every case the destruction of cystine as determined in the hydrolyzed digests was 3 times greater with trypsin than without it.



All the cystine determinations were made by the Sullivan colorimetric method.

**The Comparative Toxicity of Irradiated Ergosterol and Parathyroid Extract As Determined on the Dog and Rat.** BY JAMES H. JONES. *From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*

The comparative toxicity of irradiated ergosterol and parathyroid extract has been determined on the adult dog and rat. For irradiated ergosterol the basis of comparison was the minimum daily dose per kilo of body weight which would produce marked toxic symptoms with serum calcium of 16 mg. per cent or higher within a week. This dose has been found to be approximately 150,000 and 450,000 international vitamin D units for the dog and rat respectively. This is a ratio of 1:3. For parathyroid extract the basis of comparison has been the minimum single dose per kilo of body weight which would produce toxic symptoms with a serum calcium of 16 mg. per cent or higher within 16 hours after administration. This dose has been found to be approximately 25 units per kilo for the dog. In the case of the rat as many as 2000 units per kilo were given without producing any toxic symptoms and without any appreciable rise in serum calcium. Thus per kilo of body weight parathyroid extract is over 80 times as toxic for the dog as rat, whereas irradiated ergosterol is only 3 times as toxic. Since parathyroid extract is much less toxic for the rat than is irradiated ergosterol, it hardly seems possible that the toxicity of irradiated ergosterol is due to a stimulation of the parathyroid glands.

**The Relation of Serum Phosphate to Parathyroid Tetany.** BY JAMES H. JONES. *From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*

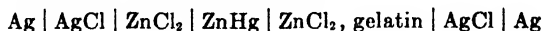
Nearly 100 per cent of parathyroidectomized rats, either young or adult, which were fed the dry portion of the Steenbock<sup>16</sup> stock diet from which the calcium carbonate was omitted, devel-

<sup>16</sup> Steenbock, H., *Science*, **58**, 449 (1923).

oped manifest tetany within 48 hours after removal of the glands. If, during a preoperative period of 3 weeks, young rats were fed this diet plus 4 per cent basic aluminum acetate, they developed a rachitic condition, with low blood phosphate. Removal of the parathyroid glands from these animals produced tetany in only a small number of cases. The animals in tetany had a high serum phosphorus and low calcium, whereas there was little or no change in the composition of the blood of the animals which experienced no tetany. Food consumption records showed that the animals with tetany had eaten practically no food following the operation as contrasted with the animals without tetany, whose food consumption was only slightly reduced as a result of the removal of the glands. In subsequent experiments animals similarly treated but deprived of food after the operation invariably developed tetany within 24 hours. It appears that when the serum phosphate was kept low, owing to the presence of aluminum acetate in the diet, the animals did not develop tetany and blood calcium remained normal even though the calcium intake was deficient. On the other hand, in the case of those animals which did not eat, the serum phosphate was increased by the starvation, with resultant decrease in serum calcium and the onset of tetany.

**Interaction of Gelatin and Salts as Measured by the Electromotive Force of Cells without Liquid Junction.** By NORMAN R. JOSEPH. *From the John Herr Musser Department of Research Medicine, University of Pennsylvania, Philadelphia*

Electromotive force determinations of salt activity have been obtained by means of cells of the type



yielding the ratio of salt activity in the presence of gelatin to that of the pure salt solution. When non-linear terms in  $m_2$ , the protein molality, are neglected, the results can be expressed by the equation

$$-\log \frac{\gamma_3}{\gamma_3^0} = \left( \frac{\alpha}{m_3^1} - \beta \right) m_2 \quad (1)$$

where  $\gamma_3$  and  $\gamma_3^0$  denote the mean ionic activity coefficient of the salt at molality  $m_3$ , the former in the presence of protein and the latter in pure salt solution.  $\alpha$  and  $\beta$  are constants.

Equation 1 can be shown by thermodynamics to be correlated with Equation 2, which expresses  $\gamma_2$ , the activity coefficient of the protein, as a function of salt molality.

$$-\log \gamma_2/\gamma_2^0 = 2\nu\alpha m_2^{\frac{1}{2}} - \nu\beta m_2 \quad (2)$$

$\gamma_2^0$  denotes the activity coefficient of the protein in pure water, and  $\nu$  represents the number of gm. ions formed by the dissociation of 1 gm. molecule of electrolyte. Such equations are frequently employed to describe the solubility of proteins in the presence of salts.

Measurements in the literature referring to salt distribution in two-phase membrane systems containing gelatin show that Equation 1 is valid for many salts,  $\alpha$  and  $\beta$  depending on the nature of the salt. In gelatin systems the values of  $\alpha$  are generally higher than those that characterize other proteins. If the results are interpreted in terms of the interionic force theory, gelatin is to be regarded as having a higher electric moment than the other proteins, including globulins, for which Equation 2 has been shown to hold.

**Concerning the Production of Anterior Pituitary Inhibitory Substances.** BY PHILIP A. KATZMAN, NELSON J. WADE, AND EDWARD A. DOISY. *From the Laboratories of Biology and Biological Chemistry, St. Louis University School of Medicine, St. Louis*

Other investigators have reported that animals become insensitive to extracts of pituitaries from heterozoic animals but do not develop this resistance when the excess of hormone is supplied by the pituitary of a homozygous animal in parabiosis. The question arises whether the inhibition results from immunological reactions or from an equilibrium between hormones and "antihormones."

In our work we have attempted to produce insensitivity to pituitary hormones in rats without involving immunological reactions by supplying the hormones in the form of rat pituitary implants.

Ten adult female rats received one to two pituitaries daily for periods varying from 7 to 9 months. During this time daily vaginal smears were made and a few laparotomies performed. At the

conclusion of the experiment the animals were killed, the endocrine glands weighed, and the serum tested for inhibitory substances.

Estrus in these animals was completely inhibited for variable periods but reappeared at very irregular intervals during the course of the experiment. In nearly all instances the ovaries remained enlarged and in no instance were they subnormal in weight. The serum from these animals did not inhibit the gonadotropic effect of rat pituitary implants in immature female rats. On the contrary, there was usually an augmentation. Furthermore, the thyrotropic action of rat pituitary implants on the immature guinea pig was not inhibited by simultaneous treatment with this serum.

Under the conditions of the experiment no evidence was obtained for the production of gonadotropic or thyrotropic inhibitory substances.

**A Physiologic and Chemical Investigation of the Suprarenal Cortex.** BY EDWARD C. KENDALL, HAROLD L. MASON, CHARLES S. MYERS, AND W. D. ALLERS. *From the Section on Biochemistry, The Mayo Foundation, Rochester, Minnesota*

Adrenalectomized dogs can be maintained in a normal condition without cortin with sodium chloride and sodium citrate. Such dogs are sensitive to many substances not ordinarily toxic; 0.5 gm. of potassium in the food as chloride, phosphate, or citrate may produce an acute crisis. With a low potassium intake dogs may be kept normal indefinitely by the administration of adequate sodium salts. Under these conditions reproduction is possible. Nine normal pups were produced by an adrenalectomized bitch mated with an adrenalectomized male dog. None of the hormone was given during the period of gestation.

Nine separate but closely related compounds are in extracts of the suprarenal cortex. These nine compounds have been separated either in original form or as degradation products or derivatives. They all have a nucleus which is composed of condensed rings. The three compounds which have the greatest significance have the following formulas:  $C_{26}H_{36}O_5$ ,  $C_{21}H_{34}O_6$ ,  $C_{21}H_{30}O_5$ .

The first compound ( $C_{26}H_{36}O_5$ ) has been identified as the precursor of an acid  $C_{20}H_{26}O_4$ . The 4 oxygen atoms are present as

one carboxyl and two ketone groups. The second compound ( $C_{21}H_{34}O_6$ ) has been described previously as a relative of glyceraldehyde. It also has a tertiary hydroxyl group and an ether group which is not methoxyl or ethoxyl. The third compound ( $C_{21}H_{30}O_6$ ) also has an aldehyde group, three hydroxyl groups, and an ether group. Rast's camphor method has been carefully checked and found satisfactory for the determination of these molecular weights. The functions of the oxygen atoms were determined in a micro-Grignard machine.

#### **A Modification of Van Slyke's Manometric Amino Nitrogen Method Yielding Theoretical Values with Cystine and Glycine.**

BY A. B. KENDRICK AND MARTIN E. HANKE. *From the Department of Biochemistry of the University of Chicago, and the Department of Medicine, the University of Illinois, Chicago*

A well known difficulty in the use of Van Slyke's amino nitrogen methods is that cystine, glycine, and other substances like glycylglycine, glutathione, and purines, give greater than theoretical values. When potassium iodide and buffered acetic acid are used in the reaction mixture, theoretical values are obtained with cystine and glycine, as well as with leucine, glutamic acid, alanine, and tyrosine.

By the use of a Harington-Van Slyke chamber, as originally suggested by Van Slyke, the entire reaction, the absorption as well as the liberation of the nitric oxide, may be carried out in the reaction chamber, and the use of the Hempel pipette thus may be avoided. This procedure is more convenient and generally reliable than Van Slyke's original method. Other less convenient methods for carrying out the entire reaction in the Van Slyke-Neill chamber have also been worked out. Any of these methods may be used with or without KI.

With urea, the KI methods yield only 1.3 per cent, compared with 7 per cent by Van Slyke's method. The urea corrections to be applied when analyzing blood filtrates directly for amino nitrogen are thus only about one-fifth as great and the KI methods are more applicable especially to uremic bloods. With blood filtrates the KI methods yield values 5 to 20 per cent lower than Van Slyke's methods. This indicates that there are present in

blood filtrates significant amounts of substances which like cystine and glycine yield too high values by Van Slyke's methods.

### **The Use of a Ten Day Period for Biological Assay of Vitamin B<sub>1</sub>.**

BY ELIZABETH KNOTT AND FREDERIC W. SCHLUTZ. *From the Bobs Roberts Memorial Hospital for Children, the University of Chicago, Chicago*

To test the validity of a short time method of assaying biological materials for vitamin B<sub>1</sub> the following procedures have been employed. (1) The duration of the depletion time (death following polyneuritis) has been checked in relation to the individual ingredients of the ration. (2) To test the validity of growth as the criterion for assay, rats have been weighed daily while receiving doses of a stabilized wheat germ, and the results compared for different levels of vitamin B ingestion. (3) The influence of the size of the animal has been determined by studying the response of rats weighing 40 to 45 gm., 50 gm., 60 to 65 gm., and 80 to 85 gm. when placed on experiment. (4) The effect of the length of the assay period on the validity of the results has been determined by comparing the amount of stabilized wheat germ causing 1 gm. gain during the 10 day periods, and during periods of 2, 4, and 8 weeks. (5) Doses of crystalline vitamin B<sub>1</sub> have been used as controls. (6) Factors causing irregularity of response have been analyzed.

### **The Epinephrine Content of Suprarenal Glands Surgically Removed in the Treatment of Essential Hypertension.** BY ALFRED E. KOEHLER. *From the Santa Barbara Cottage Hospital and the Sansum Clinic, Santa Barbara*

An estimated two-thirds to three-quarters of the suprarenal gland was removed unilaterally or bilaterally in eight cases of marked essential hypertension at ages ranging from 31 to 42 years. In the bilateral cases the second operation was performed 3 months or longer after the first stage. The preoperative basal blood pressures averaged in each case over 200 mm. of Hg systolic and 110 diastolic pressure for at least 6 months previous to operation.

The glands upon removal were immediately chilled, ground,

and extracted with 5 per cent trichloroacetic acid. The alkalinized extract was treated with iodine, the excess iodine removed with sodium thiosulfate, and the epinephrine determined colorimetrically. In several instances epinephrine was also estimated by its pressor effect after intravenous injection in rabbits.

No appreciable difference was noticed between the chemical and biological assays. The epinephrine content of the whole gland (estimated) ranged from 1.02 to 10.05 mg., with an average of 2.77 mg. The epinephrine content per gm. of tissue varied from 0.19 to 1.10 mg., with an average content of 0.52 mg. The estimated weight of the entire glands ranged from 3.595 to 9.152 gm., with an average of 4.861 gm. for eleven glands studied.

The normal human values reported in the literature are 4.0 to 5.0 gm. for the weight of each gland and 3 to 4 mg. for the epinephrine content.

Except in two cases of so called malignant hypertension the size of the suprarenal glands and the epinephrine content in essential hypertension were not greater and in some instances less than the values usually considered normal.

**The Requirement of Iron and Copper and the Influence of Diet upon Hemoglobin Formation during Normal Pregnancy.** By JEAN L. KYER AND FRANK H. BETHELL. *From the Thomas Henry Simpson Memorial Institute for Medical Research, University of Michigan, Ann Arbor*

A healthy young woman was maintained throughout the last 3 months of her first pregnancy and for 2 weeks after delivery on a uniform diet supplying a daily intake of 7.10 mg. of Fe and 2.20 mg. of Cu. The output of these elements was determined without interruption. The combined urine and stool average daily value for Fe was 7.12 mg., for Cu 2.15 mg. In spite of the absence of retention of Fe or Cu, the Hb was maintained within a range of 13.0 and 14.1 gm. per 100 cc. and the red blood cells within 4.44 and 4.80 millions per c.mm. The blood of the infant revealed high normal values. This young woman apparently possessed adequate Fe and Cu stores to supply the demands of pregnancy, including increase of the maternal blood volume and requirements of the fetus.

Studies of pregnant rats indicated that a low iron diet caused

slight anemia, characterized by hypochromia and microcytosis, whereas a low protein diet produced more severe anemia with hyperchromia and macrocytosis.

**Effects on Three Types of Animals of Injecting the New Factor Curative of Pellagra-Like Symptoms Due to Egg White.** BY JANE G. LEASE, EUNICE KELLY, AND HELEN T. PARSONS. *From the Department of Home Economics, University of Wisconsin, Madison*

Severe dermatitis and a nerve disorder in the rat due to dietary egg white were fully cured by injecting a water solution of a potent factor which is not identical with vitamin B<sub>2</sub> in any of its definitions; this is prepared by digesting Eli Lilly and Company's liver residue with papain, extracting with water, and reextracting the dried extract with methanol.<sup>17</sup> As low a level as 0.25 cc. (0.075 gm. of solid) given three times a week intraperitoneally proved to be fully curative. It was also possible to produce speedy cures when the injection was given as 1 cc. doses fortnightly.

With a chick weighing 50 to 200 gm. a 0.5 cc. injection of the potent extract given three times a week was curative of severe dermatitis of mouth and feet; it was found necessary to increase the size of dose for larger chicks. The chick has a higher requirement for the curative factor than has the rat, which might explain Franke's failure to prevent dermatitis with whole egg.

Rabbits received injections after exhibiting extensive loss of hair and marked dermatitis. 4 cc. a day relieved dermatitis and promoted extensive growth of new hair. Complications were encountered in one rabbit, and it did not survive; other rabbits are in the process of cure.

This active factor is not identical with any of the known vitamins, nor is there any apparent interrelationship with vitamin B<sub>1</sub> or B<sub>2</sub>.

**The Effect of Some Reagents on the "Filtrate Factor" (a Member of the Vitamin B Complex Promoting Growth and Preventing a Dietary Dermatitis in Chicks).** BY SAMUEL LEPKOVSKY AND THOMAS H. JUKES. *From the Division of Poultry Husbandry, University of California, Berkeley and Davis*

<sup>17</sup> *Z. Vitaminforsch.*, in press.



The name "filtrate factor" is used to refer to the member of the vitamin B complex first prepared in a fairly purified form by Elvehjem and Koehn.<sup>18</sup>

An aqueous extract of beef liver was shaken with fullers' earth and filtered. The filtrate was brought to about pH 1 and shaken repeatedly with isoamyl alcohol. The amyl alcohol was shaken with dilute sodium hydroxide. The resultant aqueous solution was neutralized and fed to chicks with a heated diet<sup>18</sup> supplemented with a fullers' earth adsorbate of whey to supply vitamin G and with hexane extract of alfalfa meal to supply the antihemorrhagic factor.

The "filtrate factor" was not precipitated by barium hydroxide in aqueous or 80 per cent alcoholic solution. It was not destroyed by bromine water, hydrogen peroxide, hydrogen sulfide, ferric chloride, sodium bisulfite, or dilute nitric acid. It was not adsorbed by acid-washed norit at pH 1 or by lead sulfide. It was partially inactivated by nitrous acid or by warming with sodium hydroxide and completely inactivated by warming with sodium hydroxide and ferric chloride. Fractional precipitation with lead acetate yielded none of the factor in the pH 6.6 precipitate, none in the pH 8.0 precipitate, and most of the potency was present in the filtrate.

Potent extracts have also been prepared by precipitating inert materials from aqueous extracts of rice bran.

Experiments have indicated that vitamin B<sub>6</sub>, described by György, may be adsorbed on fullers' earth to separate it from the "filtrate factor."

**The Blood Pressure-Raising Principle of Adrenal Cortex Extracts.\*** BY JOSEPH M. LOONEY AND MATTHEW C. DARNELL, JR. *From the Memorial Foundation for Neuro-Endocrine Research and the Research Service of the Worcester State Hospital, Worcester*

The adrenal cortex contains a principle which causes a prolonged increase in blood pressure when given by mouth. The effect is usually produced only after an extended period of medication, though certain preparations have caused a marked rise in

<sup>18</sup> Elvehjem, C. A., and Koehn, C. J., Jr., *J. Biol. Chem.*, **108**, 709 (1935).

\* This investigation was aided by a grant from Armour and Company.

4 to 5 days. On withdrawal of the medication the blood pressure drops slowly to normal in 10 days to 2 weeks.

The most active preparation was obtained by extracting whole adrenal gland with equal volumes of alcohol, glycerol, and water brought to pH 11.0 by sodium hydroxide. After removal of the glands the filtrate was made acid (pH 6.5) and the resulting precipitate removed. This filtrate was treated with permutit to remove toxic substances and concentrated *in vacuo*. 24 ml. of this solution, equivalent to 12 gm. of fresh gland, were markedly effective in raising blood pressure.

The active material is removed by precipitation by tungstic acid, phosphotungstic acid, and other protein precipitants, the filtrates from these reagents showing no potency. Much of the activity is lost during the process and only part can be recovered from the precipitate.

Some of the activity is present in the protein fraction obtained by isoelectric precipitation between pH 6.5 and 5.1.

The active principle is not soluble in ether or other fat solvents which dissolve cortin. Administration of cortical extracts, effective in Addison's disease and in maintaining adrenalectomized animals, has had no effect on blood pressure. The material is not cortin or adrenalin and the name "cortipressin" is suggested for it.

**Recent Studies on the Insuletropic Hormone.** BY A. BRUCE MACALLUM. *From the Department of Biochemistry, University of Western Ontario, London, Canada*

Failure to obtain consistently active preparations of the insuletropic hormone of the duodenum is due to the influence of a principle which is antagonistic to the hypoglycemic action of insulin and which has been found to exercise hyperglycemic effects on diabetic patients.

The action of this inhibiting factor is independent of the amounts administered, following the "all or none" law, and the effects persist for approximately 60 days after the administration of this compound.

It is stable in the presence of strong acids and will withstand boiling with 20 per cent sulfuric acid for 6 hours, indicating a simple chemical structure, but it is destroyed by boiling with 10 per cent sodium carbonate solutions for 6 hours.

Preliminary studies indicate a possible use in cases of hyperinsulinism.

**The Direct Measurement of Renal Blood Flow and Oxygen Consumption in Unanesthetized Dogs.** BY MORTON F. MASON, ALFRED BLALOCK, AND TINSLEY R. HARRISON. *From the Departments of Biochemistry, Surgery, and Medicine, Vanderbilt University School of Medicine, Nashville*

A method has been devised whereby the renal blood flow may be measured directly by collection of renal vein blood from unanesthetized dogs. Heparin is used as an anticoagulant.

The renal oxygen consumption and the renal blood flow have been determined in dogs with two kidneys, one kidney, and with one kidney explanted by the method of Rhoads.

Preliminary indirect estimations of the renal blood flow in dogs with an explanted kidney were made by the technique of Van Slyke, Rhoads, Hiller, and Alving, and these were followed by simultaneous indirect and direct observations for purposes of comparison.

Renal blood flows determined within 2 weeks after unilateral nephrectomy ranged from 13.5 to 20.9 cc. per minute per kilo of body weight with renal oxygen consumption ranging from 0.24 to 0.72 cc. per minute per kilo of body weight. Measurements on dogs with two kidneys also fell within this range. In the case of dogs with explanted kidneys, the directly measured flows were higher than those obtained by the indirect method. The rate of flow in experimental hydronephrosis is also being investigated.

**The Influence of a Diet Rich in Avocado on Growth and on the Quality of Body Fat in the Albino Rat.** BY AVA JOSEPHINE McAMIS AND MARION H. SWEET. *From the Department of Physiology, Mount Holyoke College, South Hadley*

In our studies of the relation of dietary fat to the character of body fat, we investigated the type of body fat deposited when an animal ingested *ad libitum* a large amount of a natural food of high fat content, in contrast with a synthetic diet containing a high percentage of extracted fat or oil. The avocado, unique among the fresh fruits because of its high fat content—20 per cent or more of the edible portion in some species—was chosen for this

study. Rats were grown upon a diet of fresh avocado pulp, supplemented with dried skimmed milk. Growth upon this dietary regimen compared favorably with that of rats on an adequate stock diet. The body fat of each rat was obtained by steam-rendering in an autoclave. The refractive index and iodine number (Hanus) were determined (1) on the body fat, (2) on the two samples of oil rendered from the avocado by the autoclave method, and (3) on three samples of commercially prepared avocado oil (Calavoil).<sup>19</sup>

The average value for the refractive index of the body fat was 1.468, while that of the oil was 1.470. The iodine value for the body fat averaged about 85, in contrast to the extracted oil, which was practically 10 units higher.

**The Multiple Nature of Vitamin D. III. Irradiated 22-Dihydroergosterol.** BY FRANCIS G. McDONALD. *From the Research Laboratory, Mead Johnson and Company, Evansville, Indiana*

In a study of one of the several forms of vitamin D, 22-dihydroergosterol was prepared and activated by exposure to ultra-violet rays. The active product was assayed with both rats and chickens. With rats two preparations showed, respectively, potencies of 2500 and 3100 international vitamin D units per mg. Ergosterol, irradiated under comparable conditions, had 8000 international units of vitamin D per mg. Rat unit for rat unit, irradiated 22-dihydroergosterol was found to be intermediate between irradiated ergosterol and cod liver oil in effectiveness for chickens. It resembled cod liver oil more than it did irradiated ergosterol, and was as effective as the liver oils of certain species of tuna.

**Calcification in Rachitic Cartilage Induced by Administration of Phosphate, and by Parathyroid Extract.\*** BY FRANKLIN C. McLEAN AND RICHARD H. MCCOY. *From the Physiological Laboratory of the University of Chicago, Chicago*

Uniform sublethal doses of phosphate (7.5 mg. of phosphorus per 100 gm. of body weight), in the form of a 0.1 M mixture of

<sup>19</sup> Calavoil and the avocados were kindly supplied by Calavo Growers of California.

\* This work was aided by a grant from the Josiah Macy, Jr., Foundation.

$\text{NaH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$  at pH 7.35, were injected intraperitoneally into 49 day-old rachitic rats. The serum calcium fell promptly, usually reaching a minimum of 1.6 to 1.7 mm per liter within 4 hours. Beginning calcification in the cartilage matrix was observed microscopically in the majority of the animals killed at the end of 4 hours, and almost uniformly in 8 hours, the deposit of calcium increasing in density up to 24 hours.

In rachitic rats exhibiting decalcification of bone, associated with hypercalcemia and relative hyperphosphatemia, under the influence of parathyroid extract (700 new units in 72 hours), a dense deposit of calcium in the cartilage and osteoid tissue, in the form of a typical line test, was found.

In view of previous observations to the effect that the fall in serum calcium following administration of phosphate is due to the formation of colloidal calcium phosphate in the blood and its prompt disappearance therefrom, and in view of the similarity of the conditions necessary for the formation of colloidal calcium phosphate in the blood, and for calcification, these observations, taken together with the numerous reported instances of pathological calcification under the influence of the parathyroid hormone, are consistent with the hypothesis that calcium leaving the blood and tissue fluids as colloidal calcium phosphate may be deposited in the tissues in the same form.

#### **Addition Compounds of Amino Acids with Formamide. BY**

THOMAS L. McMEEKIN. *From the Department of Physical Chemistry, Harvard Medical School, Boston*

When glycine is heated with one or more equivalents of formamide at 110–120° for 30 minutes, small amounts of ammonia and water are volatilized, leaving a heavy oil. After cooling and removing the excess of formamide with ethyl acetate, an addition compound glycine-formamide (1:1) crystallizes. The theoretical nitrogen for this combining ratio is 23.3 per cent (found 22.77 per cent). It melts between 118–125°; its aqueous solution is neutral and highly conducting, and like ammonia gives a color with Nessler's solution. Alanine and  $\alpha$ -aminocaproic acid react with formamide in a similar manner, but these addition compounds have thus far only been obtained as viscous liquids. The dipeptide glycylglycine reacts similarly with formamide.

The solubilities of the addition compounds of glycine, alanine, and  $\alpha$ -aminocaproic acid differ markedly from one another. (1) The glycine compound is insoluble in ethyl alcohol, in which the alanine and  $\alpha$ -aminocaproic acid compounds are very soluble. (2) The alanine compound is insoluble in ethyl acetate, in which the  $\alpha$ -aminocaproic acid compound is soluble, especially in the presence of excess formamide.

The amino acid formamide compounds are decomposed when heated with water or an alcohol, forming the free amino acid, ammonia, and formic acid or its ester. When glycine formamide is decomposed by butyl alcohol, a yield of over 90 per cent of pure glycine is obtained.

The properties of the addition compounds of amino acids and peptides with formamide suggest a useful method of purifying and separating amino acids or peptides from one another.

**A Study of the Cystine Content of Normal Urine.** BY GRACE MEDES. *From the Lankenau Hospital Research Institute, Philadelphia*

A study of the cystine content of normal urines has been made by the method of cuprous chloride precipitation, recovery of the cystine as cysteine by decomposition of the copper mercaptide by hydrogen sulfide, and subsequent quantitative determination of the cysteine with phospho-18-tungstic acid. Values obtained by this method in comparison with other standard methods are given.

The cystine content of 50 24 hour specimens of normal urines has been determined, together with their creatinine, total nitrogen, and neutral sulfur content, and the correlation computed between these several components.

A similar study has been made of the cystine excretion of a single individual under different metabolic conditions.

**Diffusion Coefficients of Potassium Chloride, Glycine, and Alanine.** BY JOHN W. MEHL AND CARL L. A. SCHMIDT. *From the Division of Biochemistry, University of California Medical School, Berkeley*

In a cell with a sintered glass disk, the diffusion coefficient of KCl (0.1 N KCl into  $H_2O$ ) has been determined to be 1.65 sq. cm. per day at 30°. This value agrees with the calculated value,

1.65, obtained by using the diffusion coefficient of NaCl and the cell constants determined experimentally for NaCl and KCl.

The following data were obtained: (a) glycine,  $D = 0.945, 0.94, 0.94, 0.91, 0.90$ ; (b) alanine,  $D = 0.81, 0.81, 0.80, 0.79, 0.78$ . These values correspond respectively to  $C'_0 = 0.01, 0.1, 0.5, 1.0, 1.5$ . In the above,  $D =$  diffusion coefficient in sq. cm. per day and  $C'_0 =$  initial concentration of solution, in moles per liter, in the diffusion cell.

These diffusion coefficients do not vary more than 5 per cent over the concentration range studied. The behavior of these amino acids, under the conditions of the experiment, does not deviate very much from the ideal state.

This work is being continued at other temperatures and with other amino acids.

**A New Classification of Glycoproteins.** BY KARL MEYER AND JOHN W. PALMER. *From the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, New York*

The older classification of glycoproteins must be revised. The following classification is proposed.

I. Mucins. Polysaccharide (uronic) acid and amino sugar present. (A) True mucins. Sulfuric acid absent. (1) Carboxyl group free. In nature existing at least partially as salts of inorganic bases or of basic groups of proteins. Very easily separated from protein. Examples: vitreous humor, Wharton's jelly of umbilical cord. (2) Carboxyl of polysaccharide acid combined with polypeptide, possibly as ester. Carboxyl rather easily liberated by weak acid or alkali. Example: mucin of gastric mucosa. (B) Sulfomucins. Sulfuric acid present. Protein split off only by more drastic alkaline hydrolysis. (1) Chondrosulfomucins, containing chondrosamine. Examples: cartilage, connective tissue. (2) Glucosulfomucins, containing glucosamine. Example: cornea.

II. Mucoids. Uronic acid absent, amino sugar present. Polysaccharide liberated only by very drastic alkaline hydrolysis. Examples: serum mucoid, ovomucoid, very probably the gonadotropic hormone of pregnancy urine.

III. Glucosidoproteins, having one or more sugars individually bound to protein. They may not occur in nature.

In vitreous humor only a sulfate-free polyuronic acid was found. Umbilical cord yielded a soluble polyuronic acid, resembling that of vitreous humor, and chondroitin-sulfuric acid after alkaline hydrolysis of the residue (amino sugar characterized after isolation). Commercial gastric mucin gave an acid polysaccharide by mild hydrolysis. From the sulfuric acid ester from cornea glucosamine hydrochloride was isolated. The polypeptide-sugar complex from egg white contained no sulfate, 11 per cent N, 25 per cent reducing sugar, and 8 per cent hexosamine. The gonadotropic hormone of pregnancy urine resembles in its properties the egg mucoid.

**Observations on the Polyuronic Acids of Vitreous Humor and Umbilical Cord.** BY KARL MEYER AND JOHN W. PALMER.  
*From the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, New York*

"Mucoids" usually have been obtained from vitreous humor and from other sources by precipitation of the fluid with 2 per cent acetic acid (Mörner). In many instances the products are certainly artifacts. We have found that mixtures of a neutral solution of the polyuronic acid from vitreous humor or umbilical cord with a protein solution give by this treatment artificial "mucoids." Such salts were prepared with crystallized egg albumin, edestin, and globin, having 11 to 13 per cent nitrogen and 6 to 9 per cent hexosamine. Their composition did not materially change on reprecipitation. The components singly did not precipitate with 2 per cent acetic acid.

The polyuronic acids were not dialyzable. They form long, stretched micellæ. When pressed from a fine cannula into glacial acetic acid, long doubly refractive fibers were obtained on spinning. The viscosity of the aqueous solutions was dependent upon treatment during preparation. A solution (0.25 per cent in Ringer's solution) of one substance after precipitation by glacial acetic acid had a relative viscosity of 2.17, while the same substance after precipitation by alcohol had a relative viscosity of 13.1.

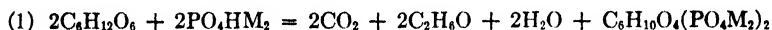
These and other similar polysaccharides are very efficient as



protective colloids: we have found it extremely difficult to remove barium or lead sulfate or metal sulfides from their solutions. This observation may explain in some instances the finding of "hydrolyzable" sulfate in such materials where barium or lead had been used in their preparation.

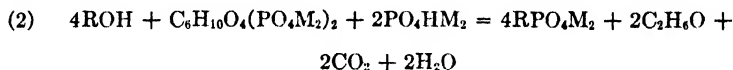
**An Improved Method of Preparing Hexosemonophosphate from Yeast Extract.** BY LEONOR MICHAELIS AND C. V. SMYTHE.  
*From the Laboratories of The Rockefeller Institute for Medical Research, New York*

The fundamental equation of fermentation in yeast extracts is the Harden-Young equation which states



According to this equation there is no monophosphate present, but, as Harden realized, the essential part of this equation is the equivalence between the phosphate esterified and the  $CO_2$  formed. Not all of this esterified phosphate is present as hexosediphosphate, as the equation shows, but a small part is present as hexosemonophosphate. This small amount of monophosphate is the source from which most preparations have been made.

In recent work by Michaelis and Smythe it was found that three dyes, naphtholsulfonate indophenol, rosinduline GG, and brilliant alizarin blue, inhibited fermentation by repressing the formation of diphosphate. If diphosphate is added to the dye containing extract, fermentation returns, but instead of the inorganic phosphate increasing, as would be expected from the straight fermentation of diphosphate, it decreases. The following equation is approximately fulfilled.



We have isolated the compound  $RPO_4M_2$  and found it to be hexosemonophosphate.

The improved method consists of allowing the fermentation to proceed according to Equation 1 until a large concentration of diphosphate is built up; the dye is then added and the fermentation allowed to proceed according to Equation 2. The monophosphate

can then be isolated in the form of a well crystallized calcium salt in much better yields than were previously obtainable.

**A Direct Microtitration Method for Blood Sugar.** BY BENJAMIN F. MILLER\* AND DONALD D. VAN SLYKE. *From the Hospital of The Rockefeller Institute for Medical Research, New York*

The proteins in 0.1 cc. of blood are precipitated with cadmium hydroxide, as described by Fujita and Iwatake,<sup>20</sup> and  $\text{BaCO}_3$  is added to remove excess Cd from solution. The filtrate is heated with a large excess of ferricyanide, and the ferrocyanide produced is titrated with ceric sulfate with setopalin C as indicator.

Only one standard solution, the ceric sulfate, is required, and only two precise measurements, one of the blood sample and one of the titration. Each 0.01 cc. of the 0.002754 N ceric sulfate indicates 1 mg. per cent of blood sugar. The blood sugar values obtained correspond closely with those for fermentable sugar.

In this procedure, direct titration of the reduction product  $\text{Fe}^{++}$  replaces estimation of reduction products from differences between initial and final  $\text{Fe}^{+++}$  or  $\text{Cu}^{++}$ , which are generally employed in current blood sugar titrations. The direct titration of the reduction product gives the present procedure the following advantages. (1) Only one standard solution is required. The ferricyanide need not be accurately measured. (2) Direct titration is more accurate than titration by difference, especially for small amounts. (3) On the other hand, since ferricyanide does not affect the titration, such a large excess can be used that the maximal amounts of sugar encountered in diabetic blood can be determined without modifying the technique. (4) The use of a large excess of ferricyanide makes the reduction exactly proportional to the amount of sugar, thereby eliminating calculation tables and curves.

**The Nitrogenous Extractives of Pecten Muscle.** BY ELINOR MOORE AND D. WRIGHT WILSON. *From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*

Further studies have been made on the guanidine compound isolated from scallop (*Pecten magellenicus*) muscle. Since Mori-

\* National Research Council Fellow in Medicine.

<sup>20</sup> Fujita, A., and Iwatake, D., *Biochem. Z.*, **242**, 43 (1931).

zawa's octopine and this compound appear not to be identical we have called it pectenine. Analyses of the free base and the picrate suggest the formula  $C_9H_{18}N_4O_4$  for the base. The molecular weight was determined to be 240. A titration in alcohol with sodium hydroxide gave an equivalent weight of 252. Methylimino and methoxy determinations were negative. The compound gave no Simon's nor nitrosamine reaction for secondary amines. These tests were also negative with proline and arginine. Hydrolysis of pectenine with barium hydroxide yielded urea and an amine,  $C_8H_{16}N_2O_4$ . Upon oxidation with  $Ag_2O$ , the amine gave about 80 per cent of the quantities of  $CO_2$  and  $NH_3$  expected from the constitution suggested below. Among the oxidation products acetaldehyde and acetic acid were identified and what seemed to be  $\gamma$ -aminobutyric acid was obtained.

These observations suggest that the compound is composed of arginine substituted on the  $\alpha$ -amino group by propionic acid attached through its  $\alpha$ -carbon atom.

From extracts of fresh scallop muscle phosphoarginine was isolated. The mother liquors yielded arginine and pectenine. In the hydrolyzed extract of fresh muscle 70 per cent of the total guanidine determined by the Sakaguchi method appeared as arginine in the arginase-urease determination. On the other hand, extracts of market muscle showed no arginine, although the total guanidine was about the same as in fresh muscle.

### Biochemical Studies on the Muscles of Dystrophic Rabbits.

By SERGIUS MORGULIS AND HOWARD C. SPENCER. *From the Department of Biochemistry, College of Medicine, University of Nebraska, Omaha*

Nutritional muscular dystrophy was produced in rabbits by means of the Goettsch-Pappenheimer Diet 13. The glycogen, creatine, acid-soluble phosphorus, and cholesterol contents of the degenerated muscles from these animals were determined. It was found that the glycogen content of the skeletal muscles was very much reduced, the reduction being proportional to the degree of degeneration, as shown by histological examination.

The total acid-soluble P was diminished in the dystrophic skeletal muscles, the decrease being again proportional to the extent of degeneration. However, even with extensive reduction

in the absolute amount of acid-soluble P, the relative distribution of the different phosphorus fractions remained practically constant. The histological examination of the muscles, together with these analytical findings, seems to indicate that the complete dissolution of individual fibers accounts for the decrease in total acid-soluble P, but that the remaining muscle fibers maintain their chemical structure intact, at least as far as the acid-soluble P compounds are concerned. The muscle creatine decreased parallel to the dystrophic change, but a greater proportion of the creatine was in the form of phosphagen in the dystrophic than in the normal muscles. The cholesterol content of the skeletal muscle greatly increased upon the onset of dystrophy, the rise in cholesterol running parallel to the course of muscle degeneration. In contrast to this, the cholesterol of the smooth muscles was only moderately increased, while in the heart, visceral organs, and brain the cholesterol concentration remained essentially unchanged.

**Preparation and Some Properties of Dried Hemoglobin.** By  
DEMPSIE B. MORRISON AND ALAN HISEY. *From the Department of Chemistry, University of Tennessee School of Biological Sciences, Memphis*

Washed cell suspensions and solutions of purified hemoglobin of dog, pig, ox, and human bloods, and suspensions of crystalline dog hemoglobin have been evaporated *in vacuo* to a final pressure of less than 5 mm. at 37–38°. The residues (which have an apparent residual water content of 3 to 6 per cent, as determined by heating to constant weight at 110°) are easily powdered and are readily soluble in water, saline, and buffers. Analyses of such solutions for total pigment and oxygen and carbon monoxide capacities (Van Slyke-Neill) and spectrophotometric measurements, demonstrate that the solute is active hemoglobin and that the principal, if not sole, pigment contaminant is methemoglobin. Some preparations have shown an oxygen or carbon monoxide capacity of 98 to 100 per cent of the theoretical; there is no consistent relationship between the activity and residual moisture content of the various samples. The dried preparations, which are apparently reduced hemoglobin, are converted to methemoglobin when exposed to air but are stable when sealed *in vacuo*. The oxygen uptake of the powder, measured in the Warburg apparatus, is rapid and is not accompanied by carbon dioxide production.

**Dehydration As a Factor Influencing the Concentration of Serum Chloride, Base, and Protein of the Normal Dog during Exercise.**

BY MINERVA MORSE AND FREDERIC W. SCHLUTZ. *From the Bobs Roberts Memorial Hospital for Children, the University of Chicago, Chicago*

A study has been made of the effect of the water loss which occurs as a heat-dissipating mechanism during exercise upon the concentration of chloride, base, and protein in the blood serum of the normal dog.

The rate of dehydration, as determined by loss of weight, and the rate of rise of body temperature were found to increase as conditions were imposed which reduced the length of time the dog was able to run, such as increasing the incline, the speed, or the room temperature.

Dehydration in itself did not appear to be a cause of fatigue.

The concentration of serum chloride and base increased with treadmill exercise, the chloride to a greater degree than the base. The degree of increase of both varied with the degree of dehydration. The concentration of serum chloride at exhaustion corresponded fairly closely with that calculated as the result of dehydration; the concentration of serum base was less than the calculated value by as much as 11 mm. The results suggest that during long continued exercise base is lost, but the concentration of serum chloride in the dog is determined largely by the extent of dehydration.

The concentration of serum protein increased with exercise, but the increase bore no apparent relation to the degree of dehydration. The results are interpreted in terms of a balance between osmotic and hydrostatic forces.

**The Vitamin C Content of Tumor Tissue.** BY R. R. MUSULIN, GLADYS E. WOODWARD, ETHYL SILVERBLATT, AND C. G. KING. *From the Department of Chemistry, University of Pittsburgh, Pittsburgh, and The Biochemical Research Foundation of the Franklin Institute, Philadelphia*

3 week rat tumors, Philadelphia No. 1 sarcoma, relatively free from necrotic areas and high in ascorbic acid content, were kept in dry ice during intervals of 2 to 5 days without a significant loss in indophenol titration value. Finely crushed samples were titrated

chemically by the method of Bessey and King, modified by adding 2 per cent metaphosphoric acid, and fed in standard suspension to guinea pigs in quantities corresponding to 0.25 and 0.50 mg. of vitamin C per day. The growth response and degree of protection from scurvy agreed with the titration value within the limits of biological assay, in comparison with animals receiving a standard solution of the pure vitamin. The indophenol titrations were further checked by destruction of the ascorbic acid by means of the specific oxidase described by Tauber and Kleiner. In this case, the dye titration, but not the iodine titration, approached zero at a rate corresponding with vitamin C oxidation.

**The Potassium Content of Muscle and Its Possible Relation to Muscle Creatine.\*** BY VICTOR C. MYERS AND GEORGE MANGUN. *From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland*

In 1922 one of us<sup>21</sup> stated that "there are many observations which lead one to believe that glycogen, creatine, phosphoric acid and potassium are associated in active muscle." In connection with studies<sup>22</sup> of the creatine content of rabbit muscle under varying conditions, estimations of P and K were carried out by one of us. A parallelism was noted between the creatine and potassium at the time, but seemed difficult of interpretation. Fiske and Subbarow<sup>23</sup> had not yet discovered phosphocreatine. Recently the creatine, potassium, and phosphorus content of human voluntary and heart muscle has been studied. A few observations have also been made upon the voluntary muscle of the guinea pig and dog. With creatine as the compound of reference it has been found that K holds a fairly constant ratio in the same and different species. Considering the creatine as phosphocreatine, the K is somewhat more than sufficient, for example, to satisfy the 2 free hydrogens of the phosphoric acid, the figures for the rabbit, guinea pig, and man averaging in each

\* Aided by a grant from the Josiah Macy, Jr., Foundation.

<sup>21</sup> Myers, V. C., in Barker, L. F., *Endocrinology and metabolism*, New York and London, 3, 463 (1922).

<sup>22</sup> Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 15, 283, 305 (1913); 16, 169 (1913).

<sup>23</sup> Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 81, 629 (1929).

species 133 per cent of this hypothetical requirement. In the case of heart muscle there is a considerably greater excess in K over the formula requirement (223 per cent), but this excess in relation to the creatine is the same in both left and right ventricular muscles, although they differed in creatine content by 28 per cent. Again calculating the creatine as phosphocreatine and taking this as the compound of reference, it has been found that in voluntary muscle (human, guinea pig, rabbit, dog) the P is almost exactly twice this hypothetical requirement, while in heart muscle it is 4 times this amount. What significances these ratios may have, if any, remain to be demonstrated.

### **The Hemoglobin Concentration and Red Cell Count of Healthy**

**Men.** BY C. FERDINAND NELSON AND RUTH STOKER. *From the Department of Biochemistry, University of Kansas, Lawrence*

Data on the normal values for hemoglobin concentrations and red cell counts in healthy men have, in the past, been collected almost exclusively from doctors, medical students, laboratory workers, and attendants in and about hospitals and medical schools. The bulk of the data has come also from men under 30 years of age.

Oxygen capacity determinations and red cell counts have been made on blood obtained from 325 healthy men from other walks of life. The subjects chosen include groups of urban and suburban mail clerks and mail carriers, traffic and motor cycle policemen, young men in civilian conservation corps camps, soldiers from the regular army, including groups from the infantry, cavalry (colored), medical corps, motor transport corps, and air corps.

The values obtained for the age range studied (18 to 65 years) were: for hemoglobin 11.93 to 17.92 gm. per 100 cc., with a mean of 14.38; for red cells 4.03 to 6.70 millions per c.mm., with a mean of 5.12.

It is suggested that hemoglobin and red cell standards be expressed not only in absolute units, as is now customary in many laboratories, but that the single average or arithmetical mean values now so often used also be abandoned for the more significant and clinically valuable maximum and minimum values. Any single standard value, whether it be expressed in absolute or relative units, that is not also an optimum value becomes a "dummy"

standard quite as truly as any of the existing percentage standards now in use and against which so much justifiable criticism has recently been raised.

**Vitamin E and Early Growth.** BY H. S. OLCOTT AND H. A. MATTILL. *From the Biochemical Laboratory, State University of Iowa, Iowa City*

Several investigators have suggested that vitamin E has metabolic functions other than that of conferring fertility. The following experiments were designed to demonstrate the growth-promoting ability of a diet rigorously freed of vitamin E. This diet consisted of casein (alcohol- and ether-extracted) 20, yeast<sup>24</sup> (ether-extracted) 10, sucrose 61.5, distilled ethyl esters of mixed fatty acids (from hydrogenated cottonseed oil) 4, salt mixture<sup>25</sup> 4.5, and calciferol<sup>26</sup>  $1 \times 10^{-7}$  parts, and was supplemented twice weekly with carotene.<sup>26</sup> Rats fed from weaning on this diet demonstrated growth equal to or better than that of similar animals on a stock colony ration. Supplements of highly concentrated vitamin E fractions from cottonseed oil or palm oil, begun at weaning, did not increase the rate of growth in females but did slightly in males. Small supplements of wheat germ oil, sufficient to insure fertility, had little if any favorable effect on either sex. The inclusion of lard in the diet induced in both sexes a growth which was significantly better than that of the rats on the control diets. Both males and females on the control diets and on those containing lard were entirely sterile at 130 days. Those receiving vitamin E supplements were fertile. Such improvement in early growth as has been reported following the use of wheat germ oil is apparently not due to its vitamin E content. The responses of older animals are under observation.

**Studies in the Physical Chemistry of Lipopeptides. I.** BY JOHANNES VAN ORMONDT. *From the Department of Physical Chemistry, Harvard Medical School, Boston*

The spreading of proteins on surface layers has been shown to depend not only on the shapes of molecules, but also on the polarity

<sup>24</sup> Courtesy of the Northwestern Yeast Company.

<sup>25</sup> Hawk, P. B., and Oser, B. L., *Science*, **74**, 369 (1931).

<sup>26</sup> Courtesy of Mead Johnson and Company.



of their chemical groups. Whereas most proteins can be spread on a surface film, amino acids and the simpler peptides are too soluble as a result of their zwitter-ionic structure and relatively short paraffin chains. In order further to investigate the forces due to the polar groups of amino acids, peptides, and proteins, and to account for the surface area of proteins in terms of the surface of those groups, lipopeptides have been synthesized, the length of whose paraffin chain was sufficiently great to render them far more soluble in organic solvents than in water.

Two series of compounds were prepared. The first is of the type  $R_1\text{CONH}\cdot\text{CHR}_2\cdot\text{COOH}$ , in which  $R_2$  is either  $\text{C}_4\text{H}_9$  or  $\text{C}_6\text{H}_{13}$ , and  $R_1$  is varied from  $\text{C}_{11}\text{H}_{23}$  to  $\text{C}_{18}\text{H}_{37}$ , and contains a peptide linkage and a carboxyl group. These compounds were prepared by the interaction of  $\alpha$  and amino acids and the acid chlorides of fatty acids. The compounds of the second series differ from the first in that they contain an  $\text{NH}_2$  group at the  $\alpha$ -carbon atom of the group  $R_1$ , and are thus zwitter ions of different electric moments. In their preparation instead of the fatty acid chlorides, the acid chlorides of the  $\alpha$ -bromo fatty acids were combined with the amino acids and afterwards the bromine replaced by the amino group.

The monomolecular films of these molecules are being investigated, as well as their viscosities, solubilities, and electric moments, not only in water but in organic solvents.

**Precursors of Endogenous Citric Acid.** BY JAMES M. ORTEN AND ARTHUR H. SMITH. *From the Department of Physiological Chemistry, Yale University, New Haven*

A number of known metabolites and related compounds have been studied as possible precursors of endogenous citric acid in dogs fed a "synthetic," citrate-low ration. After a 10 day control period on the basal diet, the animals were injected daily for 3 days with the substance under investigation and then treatment was suspended for a control period of 5 days. The same procedure was repeated for each substance tested. The compounds were injected intravenously, usually as 5 per cent solutions of the sodium salts, in such amounts that each animal received 100 mg. of sodium per kilo of body weight. The daily urinary citric acid excretion was determined by a photometric method.

The results thus far obtained indicate that the animals excrete from 4 to 12 mg. of citric acid daily during the control periods and that the injection of sodium chloride does not significantly alter this value. Sodium bicarbonate, lactate, and acetate produce increases in urinary citric acid to from 2 to 6 times the amount excreted during control periods, and simultaneous increases in urinary pH. Sodium malate, succinate, and fumarate cause a 40- to 50-fold increase in citric acid excretion, with a less pronounced shift in urinary pH. Sodium tartrate, on the other hand, does not increase the citric acid excretion more than can be accounted for by its sodium content. The effects of sodium acetoacetate, butyrate, aspartate, glutamate, and glycinate are now under investigation.

**Factors Influencing the Distribution and Character of Adipose Tissue in the Rat. III. The Effect of Thyroglobulin and of Thyroxine.** BY FELIX PAQUIN, JR., AND WILLIAM E. ANDERSON. *From the Department of Physiological Chemistry, Yale University, New Haven*

The influence of thyroid material, administered *per os*, was studied in male and female rats fed diets equicalorically rich in either coconut oil or carbohydrate (corn-starch). Thyroglobulin was given to young rats, whereas thyroxine was first fed to older rats of 200 gm. in body weight.

The thyroid hormone produced a decrease in gain or a loss in body weight in animals of both age groups. The percentage of body fat obtained from different locations in the body was influenced by the administration of thyroid material. In the young animals grown during a period of hyperthyroidism, the proportion of fat in the subcutaneous and intermuscular depots was increased; the proportion of fat in the perirenal and genital depots was decreased; while that of the mesenteric and omental depots underwent no significant change. A qualitatively similar picture of fat deposition and distribution was produced in animals grown by restriction of food intake at the same rate and to the same age as the thyroglobulin-fed animals, with the exception that the proportion of genital fat was not so markedly diminished. Apparently the changes in the hyperthyroid animal were associated with diminished growth rate.

The administration of thyroxine to young adult animals increased the proportion of subcutaneous and intermuscular fat, diminished the proportion of genital and perirenal fat, and did not appreciably alter the proportion of mesenteric and omental fat.

**Chemical Investigations on the Cortical Hormone of the Adrenal Gland.** BY J. J. PFIFFNER AND OSKAR WINTERSTEINER.

*From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

The active concentrates obtained by distribution procedures previously described have proved to be highly complex mixtures. The isolation of five crystalline, physiologically inactive compounds was reported last year. None of these compounds absorbs light in the ultra-violet region, while the highly active syrup remaining after separation of these substances exhibits strong selective absorption at  $240\text{ m}\mu$ . We have found this absorption to be partly due to a crystalline compound  $\text{C}_{21}\text{H}_{28}\text{O}_6$  melting at  $217^\circ$  (uncorrected). This compound also has been found to be physiologically inactive in the adrenalectomized dog. 2 of the oxygen atoms are present as carbonyl groups and at least 1 as hydroxyl.

The active material remaining in the mother liquor shows absorption in the same region and of almost the same intensity as the new compound, but contains a smaller number of carbonyl groups per unit weight.

**Experiments on the Distribution and Renal Excretion of Sucrose Injected Intravenously in Dogs.** BY MARSCHELLE H. POWER AND NORMAN M. KEITH. *From the Section of Clinical Metabolism and Division of Medicine, The Mayo Clinic and The Mayo Foundation, Rochester, Minnesota*

The sugar was administered in 20 per cent solution in amounts varying from 0.12 to 1.0 gm. per kilo. It appears quickly in thoracic duct lymph, and soon assumes a concentration comparable to that in the plasma. After injection of large amounts of more concentrated solutions of sucrose, it was found in considerable quantity in all tissues examined, being especially concentrated in the liver and kidney.

When sucrose is injected into men in amounts ranging from 0.07 to 1.0 gm. per kilo, it is almost completely excreted in the urine, indicating that little or no inversion occurs.<sup>27</sup> However, in the dog, only 70 to 80 per cent of the quantity injected appears in the urine. At the same time considerable reducing sugar is also excreted. Chemical and polarimetric examinations indicate that this reducing sugar is largely levulose. This may be taken as objective evidence that in the dog inversion has occurred. Whether this occurs in various tissues or in the blood only cannot be stated. Although numerous investigators have failed to find invertase in dog blood, the recent successful demonstration of its presence by Abderhalden and Buadze following sucrose injection suggests that at least part of the inversion takes place in the blood stream.

**Oxidation-Reduction Potentials of N-Methyl- $\beta$ -Oxyphenazine (Isomer of Pyocyanine) and Related Substances.** BY PAUL W. PREISLER AND LOUIS H. HEMPELMAN. *From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis*

The oxidation-reduction potentials of  $\beta$ -oxyphenazine and of N-methyl- $\beta$ -oxyphenazine have been determined.

In dilute HCl both show two-step oxidation-reduction, passing from the yellow, fully oxidized forms through intermediate greens to the pale yellow or colorless reductants. In neutral and alkaline solutions the potential titration curves are essentially 2 valent in form. At biological pH the N-methyl derivative is deep red in color and the  $E'_0$  values lie about midway between those of rosinduline and pyocyanine. The  $\beta$ -oxyphenazine is orange-yellow in color. At biological pH its  $E'_0$  curve lies between that of rosinduline and  $\alpha$ -oxyphenazine; in the more alkaline region, because of an ionization, its  $E'_0$  curve turns toward the hydrogen electrode and is more negative than other indicators now known.

Except where low solubility in certain pH regions is objectionable, these substances may be useful oxidation-reduction indicators.

<sup>27</sup> Keith, N. M., Power, M. H., and Peterson, R. D., *Am. J. Physiol.*, **105**, 60 (1933).

**The Coagulation Defect in Peptone Shock and in Sweet Clover Disease.** BY ARMAND J. QUICK. *From the Department of Pharmacology, Marquette University School of Medicine, Milwaukee*

The linear relationship which has been found to exist between the clotting time of plasma and the concentration of thrombin is destroyed when heparin or various types of antithrombic agents are added to plasma, thereby furnishing a delicate means for detecting the presence of antithrombin. Normal human, dog, and rabbit plasmas were found to contain no demonstrable amounts of heparin. Dog plasma made incoagulable by the intravenous injection of peptone exhibited a marked antithrombic action when tested with progressive dilutions of thrombin. Thus, a concentration of thrombin which coagulated normal plasma in 7 seconds was found completely inactivated when added to plasma obtained after the injection of peptone. No significant diminution of prothrombin was observed.

After feeding spoiled sweet clover hay to rabbits, a gradual diminution of prothrombin occurred as measured by the author's method for the quantitative estimation of prothrombin. Rabbit plasma which normally clots in 7 to 9 seconds when excess thromboplastin and calcium are added exhibited a clotting time of 20 seconds after the animal had been fed spoiled sweet clover for a week. This constitutes a prothrombin depletion of 75 per cent, bringing it to a level at which serious hemorrhages may occur. This plasma when tested with progressive dilutions of thrombin clotted as readily as normal plasma, demonstrating the absence of antithrombin. Since a toxic substance can cause a diminution of prothrombin, the possibility exists that the hemorrhagic diathesis observed in certain types of jaundice may be due to a toxin, for in the latter condition a deficiency of prothrombin has likewise been found.

**Clinical and Metabolic Studies of the Eskimo.** BY I. M. RABINOWITCH. *From the Department of Metabolism, the Montreal General Hospital, Montreal, Canada*

A clinical and laboratory study was made of the Eskimo during the Canadian Eastern Arctic Patrol of 1935. Clinical studies included general physical examinations, ordinary urinalyses, and

x-ray and hematological studies. Metabolic data included determination of non-protein nitrogenous constituents of blood (urea, creatinine, uric acid, amino acids, etc.), respiratory metabolism, blood sugar time curves, chemical and spectrophotographic analyses of the inorganic composition of urine, chemical composition of food materials of the Eskimo, and analyses of Arctic waters at twenty-five different latitudes and longitudes. Of special interest, physiologically, are the high non-protein nitrogenous content of the blood from subjects with no obvious renal disorders, improper utilization of ingested carbohydrates, and, with respect to formation of hemoglobin, the relationship between the high incidence of polycythemia among these people and the copper content of their food materials. From the respiratory metabolism data, it would appear that, in the Eskimo, may be found the solution to the question of the conversion of fat to sugar.

**A Fermentable, Zinc-Precipitable Reducing Substance in Blood in Diabetic Coma.** BY JOHN G. REINHOLD AND T. V. LETONOFF. *From the Biochemical Division, Pathological Laboratories, and the Metabolic Division, Philadelphia General Hospital, Philadelphia*

It was found by one of the writers (T. V. L.) that reduction of alkaline copper solutions by protein-free filtrates of oxalated blood taken from patients in diabetic coma is considerably greater when filtrates are prepared by the use of tungstic acid as compared with filtrates prepared by means of a zinc hydroxide powder precipitant.<sup>28</sup> Differences as great as 150 mg. per cent (as glucose) have been observed. The difference disappears within a few hours following treatment with insulin and other agents used to combat diabetic coma. It has now been shown by fermentation of the tungstic acid filtrates that concentrations of non-fermentable reducing material approximate those of normal blood. Therefore, the greater part of the zinc precipitate consists of a fermentable substance. The latter is present in both cells and plasma, although concentrations are greatest in plasma.

The material is thought to be a phosphoric acid ester. It is known that certain phosphoric acid esters formed in the course

<sup>28</sup> Letonoff, T. V., *J. Biol. Chem.*, **106**, 693 (1934).

of carbohydrate metabolism are fermentable and capable of reducing alkaline copper solutions. Tests have shown that hexosediphosphoric acid (Harden-Young) is precipitated by zinc hydroxide and other zinc salts. On the other hand, glucose-6-monophosphoric acid<sup>29</sup> is not precipitated. Studies of blood phosphorus support the belief that the material in question is a phosphorus compound.

**Studies on the Acid-Base Condition of Blood. VI. The Changes in Color and Absorption Curves of a Phosphate Buffer-Phenol Red Solution on the Addition of Blood Serum of Various Species.** BY HOWARD W. ROBINSON, J. WAIDE PRICE, CORINNE HOGDEN, NORTON NELSON, AND GLENN E. CULLEN. *From the Children's Hospital Research Foundation and the Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati*

Blood serum is diluted 1:20 in physiological saline in the colorimetric pH method. However, it is more satisfactory to study the influence of serum on the color of phenol red in a serum-dye-buffer system. When 1 part of serum was added to 20 parts of m/15 phosphate solution containing phenol red, the absorption curve of the indicator was altered to a different extent by human, rabbit, and pig sera. Dog sera had little effect. Parallel pH determinations, colorimetrically, electrometrically with a glass electrode, and spectrophotometrically (with density values for 565  $m\mu$ ) agreed only in the case of dog sera.

In the colorimetric determinations there was a "color loss" equivalent to 0.10 pH with rabbit serum, to 0.12 with normal human serum, to 0.08 with one goat serum. Dog serum had no "color loss," while the sera of two young pigs caused a "color increase" of 0.04 and 0.07 pH. These differences of color readings are explained by the absorption curves. The effect is due mainly to the albumin fraction of the protein. At pH 11.5 ( $\text{Na}_3\text{PO}_4$ - $\text{Na}_2\text{HPO}_4$ ) there was no effect on the color by rabbit serum. The ultrafiltrate from rabbit serum had no effect on the color.

With human serum the "color loss" is less in a sodium chloride-serum-dye system than in a phosphate-serum-dye system.

<sup>29</sup> Hexosephosphate preparations were obtained from The Biochemical Research Foundation of the Franklin Institute, Philadelphia, through the courtesy of Dr. Schroeder.

**The Present Status of the Amino Acids in Nutrition.** BY WILLIAM C. ROSE, KENNETH S. KEMMERER, MADELYN WOMACK, EDWIN T. MERTZ, J. KENNETH GUNTHER, RICHARD H. MCCOY, AND CURTIS E. MEYER. *From the Laboratory of Biochemistry, University of Illinois, Urbana*

In continuation of the investigations in this laboratory, involving the use of synthetic mixtures of amino acids in place of proteins, it has been shown that methionine is an indispensable component of the food. Diets entirely devoid of cystine, except for the traces which may be furnished by the vitamin supplements, support rapid growth, provided methionine is present. On the other hand, when cystine is furnished in place of methionine, the animals fail to grow, or do so at greatly diminished rates. Evidently, cystine can function for methionine to a very limited extent, if at all.

Animals upon diets devoid of glutamic acid, hydroxyglutamic acid, proline, hydroxyproline, and arginine, but containing all other constituents of a normal ration, grow slightly less rapidly than do controls which receive our complete amino acid mixture. The data may indicate that the five amino acids in question cannot be synthesized *simultaneously* by the organism at sufficiently rapid rates to meet the requirements of normal growth. This possibility is being further investigated.

Glycine and serine have been shown to be unnecessary for growth. It should be pointed out that certain amino acids which ordinarily are not required may become necessary for detoxication purposes when foreign materials with which they conjugate are included in the food.

Further information has been obtained concerning the spatial configuration of *l*- $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric acid.

**Simultaneous Studies of the Colloid Osmotic Pressure of the Blood Serum and Changes in Erythrocyte Form during Pregnancy.** BY ELLEN SCHOCK ROTTSCHAEFER AND FRANK H. BETHELL. *From the Thomas Henry Simpson Memorial Institute for Medical Research, University of Michigan, Ann Arbor*

The colloid osmotic pressure of the blood serum has been determined directly in fifteen normal pregnant women by the method of Wells and the results obtained have been correlated with the



albumin and globulin content of the same samples. In most instances repeated observations were made on the same subject throughout the latter months of their pregnancies and in a few cases plasma volume determinations were made. Mean erythrocyte volume and diameter were determined in all cases with the same samples of venous blood as those used for the serum studies. In subjects with progressive lowering of the serum colloid osmotic pressure coincident with increasing blood dilution it was found that the erythrocytes assumed a more spherical form, as indicated by normal or slightly increased average cell volume and definitely decreased average cell diameter. When hemoglobin deficiency was present, these changes were masked by the resulting microcytic anemia.

**The Rapid Thermochemistry of Carbonic Acid.** BY F. J. W. ROUGHTON. *From the Physiological Laboratory, University of Cambridge, Cambridge, England*

The rôle of carbamino compounds in the respiratory transport of  $\text{CO}_2$  by the blood has until now been investigated by equilibrium studies on the amount of  $\text{CO}_2$  bound to hemoglobin in the carbamino form under physiological conditions. The next stage is to study the kinetics of the process of  $\text{CO}_2$  combination both with (a) hemoglobin, (b)  $\text{H}_2\text{O}$  to form  $\text{H}_2\text{CO}_3$  (under the influence of carbonic anhydrase) *in whole blood*. For this purpose the rapid thermal method of Bateman and Roughton appears well suited, but as a preliminary it is necessary to know the heats of the separate reactions (1)  $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$ , (2)  $\text{H}^+ + \text{HCO}_3^- \rightarrow \text{H}_2\text{CO}_3$ . These have not been separately measured by the usual calorimetric methods, since these give only the sum of the heats of reactions (1) and (2). By the aid of the rapid thermal method it has been found that the heat of reaction (1) is 1600 calories and of reaction (2) 1300 calories at  $19^\circ$ . The velocity constant of the reaction  $\text{H}_2\text{CO}_3 \rightarrow \text{CO}_2 + \text{H}_2\text{O}$  has also been measured by the thermal method, and checks well with the values given by other rapid methods.

**Synthesis and Destruction of Organic Molecules in the Animal Organism As Measured with Deuterium As an Indicator.** By RUDOLF SCHOENHEIMER AND D. RITTENBERG. *From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

Organic molecules synthesized in a medium of heavy water may contain deuterium atoms which are not exchangeable with hydrogen atoms of water. When the body fluids of mice on a bread diet were brought to an increased concentration of heavy water, the fatty acids (both saturated and unsaturated) of the animals were found to contain considerable amounts of "stable" deuterium, a maximum being reached after 6 to 8 days. The unsaturated fraction, on oxidation, yielded azelaic acid containing deuterium in the same concentration as in the original unsaturated acids. As in mammals there are almost certainly no fatty acids which contain double bonds in the fragment represented by the azelaic acid, the deuterium could not have entered it by saturation of double bonds present in positions in which they exist in unsaturated acids of mammalian origin.

Since the animals did not change their weight, the observed synthesis must have been accompanied by a simultaneous breakdown. To demonstrate the destruction of fatty acids, the bread diet, with ordinary water, was supplemented by deuterium-rich fatty acids, which the mice deposited in their fat tissues. After the body fats were thus labeled, the fatty acids were withdrawn, and the mice were continued on a diet of bread alone. Deuterium disappeared from the body fats at about the rate observed for the formation of deuterium fats in the first experiment.

Similar results were obtained with cholesterol.

**An Aeration Method for Determining Lactic Acid.** By ALEITA HOPPING SCOTT. *From the Department of Physiology, College of Physicians and Surgeons, Columbia University, New York*

The method consists in an oxidation of the lactic acid by permanganate and aeration through Folin ammonia absorption tubes, collecting the aldehyde in bisulfite solution.

Two burettes are used in the final titration. Special tips are used, so that the iodine and the bicarbonate may be introduced simultaneously into the titration mixture. The bicarbonate (10

per cent) is added drop by drop. The titration is made in an excess of iodine; *i.e.*, the solution is never allowed to become colorless. Underneath lighting is used in determining the end-point. 1 drop of 0.001 N iodine makes a perceptible color change.

Dilute iodine is made in 5 per cent KI in order to reduce the titration error of the dilute iodine solutions and to increase their stability.

In order to reduce the air oxidation of bisulfite, Merck's reagent bisulfite is used. Also, all of the solutions that are used in the titration are made in especially prepared distilled water that is kept in a silver-lined tank to prevent the presence of metallic catalysts. The tubes are cooled to about 2° just before titration.

Blanks are found to vary with the dilution of the iodine.

Recoveries of known quantities of Zn lactate (0.725 mg.) were  $99.5 \pm 1.6$  per cent (mean deviation) on ten determinations. Recoveries of lactate added to the blood were of the same order.

**The Prolongation of Insulin Action by Protamine and Zinc.** By D. A. SCOTT AND A. M. FISHER. *From the Connaught Laboratories, University of Toronto, Toronto, Canada*

Following the lead provided by Hagedorn and his collaborators, samples of protamine have been prepared from the testes of two species of fish (*Oncorhynchus tshawytscha* and *Oncorhynchus kisutch*). The process used was largely that described by Kossel. The preparations were then purified, reducing the ash content by precipitation from hydrochloric acid with alcohol, and by electro-dialysis. Samples of these preparations were mixed with commercial, crystalline, or amorphous insulin of low ash content and the mixtures were injected subcutaneously into rabbits. The level of the blood sugar was determined for a period of at least 10 hours after the injection. The results showed that hypoglycemia was more prolonged in those rabbits which had been injected with a mixture prepared several days previously than in those receiving freshly prepared material. The addition of a small amount of zinc chloride to the insulin solution before combination with the protamine resulted in a more prolonged insulin action than was observed when the protamine-insulin without added zinc was injected. These results suggest the possibility

that zinc or other metals may play a rôle in the union of insulin and protamine. Attempts were made with beef pancreas to prepare other substances which might prolong the action of insulin. Spermine from this source was found to behave somewhat similarly to protamine.

**Acid-Base-Combining Capacity of Tuberculin Protein.** BY FLORENCE B. SEIBERT. *From the Henry Phipps Institute, University of Pennsylvania, Philadelphia*

Various highly purified tuberculin protein fractions with corresponding tuberculin potencies, when prepared in different ways, some even electrodyalyzed to constant conductivity, vary markedly in their acid-base-combining powers, as shown by means of electrometric titration, with the hydrogen electrode referred to a saturated calomel half-cell. For example, a fraction isolated from unheated tuberculin by half saturation with ammonium sulfate has practically the same buffering capacity as crystalline ovalbumin. It binds much less alkali, however, than fractions which have been heated or treated with acid or alkali. The extent to which the fractions are able to bind alkali varies inversely with their antigenic capacities, and also with their molecular sizes as determined previously by means of osmotic pressure determinations. Significant differences as well as similarities in acid-base-combining capacities and in pH range of precipitability have been found between purified fractions of protein made in different ways from the same strain of tubercle bacilli, on the one hand, and between purified proteins made in the same way from different strains of acid-fast bacilli on the other. These results are of value in studies involving further purification and separations and in explaining the relative stability of the solutions and the composition of the active constituent.

**Studies in Carcinogenesis. III. Isomers of Cholanthrene and Methylcholanthrene.** BY M. J. SHEAR. *From the Office of Cancer Investigations, United States Public Health Service, Harvard Medical School, Boston*

In further experiments in the investigation of carcinogenic compounds, which is being carried on jointly with Professor L. F. Fieser and his colleagues of the Division of Chemistry of Harvard

University, it was found that 8,9-dimethylene-1,2-benzanthracene was less active than cholanthrene, of which it is an isomer. On treatment with cholanthrene, tumors appeared in one half of the mice during the 3rd month and in the other half during the 4th month. The isomer produced during the first 4 months only one tumor in fourteen mice; another tumor appeared during the 5th month, and four more during the 6th month.

Likewise, 7-methyl-8,9-dimethylene-1,2-benzanthracene was less active than methylcholanthrene, of which it is an isomer. With methylcholanthrene, tumors appeared in almost all of the mice during the 3rd and 4th months, whereas with the isomer no tumors were obtained in 11 months.

While the methylcholanthrene has about the same activity as cholanthrene, addition of another methyl group to form 16,20-dimethylcholanthrene results in a reduction of activity, as shown by a definite increase in the latent period.

Similarly, while 4'-methyl-1,2-benzpyrene is strongly carcinogenic, it has a longer latent period than benzpyrene. These results are analogous to those obtained by the English workers on introducing methyl groups into the molecules of active compounds.

1',9-Methylene-1,2,5,6-dibenzanthracene, which is related structurally to both methylcholanthrene and 1,2,5,6-dibenzanthracene, is also carcinogenic but the latent period is at least as long as that of dibenzanthracene.

**Studies in Carcinogenesis. IV. Development of Liver Tumors in Pure Strain Mice Following the Injection of 2-Amino-5-Azotoluene.** BY M. J. SHEAR. *From the Office of Cancer Investigations, United States Public Health Service, Harvard Medical School, Boston*

Since 1931 Yoshida has published in the "Transactions of the Japanese Pathological Society" a series of papers on the occurrence of atypical regeneration of epithelial tissue in experimental animals following the administration of a compound designated as *o*-amido-azotoluene; in many instances the induced proliferation resulted in tumor formation.

This compound has been injected, as a solid, in the subcutaneous tissue of pure strain mice. 1 year after the first injection, four

mice were found to have multiple tumors in the livers. Some of the negative mice are still alive.

The tumors obtained in this way were liver cell carcinomas. Thus the carcinogenic action of this compound is confirmed.

**Differentiation of Glucosides by Prolonged Action of Bromine Involving the Second Stage of Oxidation.** BY FAY SHEPPARD AND MARK R. EVERETT. *From the Department of Biochemistry, University of Oklahoma Medical School, Oklahoma City*

1 per cent aqueous solutions of  $\alpha$ - and  $\beta$ -glucosides, oxidized with bromine and aerated, differ in optical behavior and naphthoresorcinol tests. Differentiation by the naphthoresorcinol test is limited to aldohexosides, while optical rotation is more generally applicable.  $\alpha$ - and  $\beta$ -disaccharides can be differentiated by naphthoresorcinol only after oxidation at room temperature, and polysaccharides not at all. Both disaccharides and polysaccharides can be differentiated by optical behavior, whether oxidized 6 weeks at 25° or 4 hours at 65°.

Optical rotations of oxidized  $\beta$ -methyl hexosides or pentosides approximate those of the corresponding hexoses or pentoses similarly oxidized.  $\beta$ -Glucosides are hydrolyzed and sufficient keturonic acids are formed to cause intense blue naphthoresorcinol tests with oxidized  $\beta$ -aldohexosides. Obviously,  $\beta$  substitution on carbon atom (1) introduces little hindrance to the addition of bromine to the cyclic oxygen.

Oxidized  $\alpha$ -methyl glucosides and  $\alpha$ -polysaccharides show comparatively little change of rotation (less than 10 per cent), but reducing  $\alpha$ -disaccharides exhibit a little more (less than 20 per cent), owing to bionic acid formation during the first stage of oxidation. The  $\beta$ -disaccharides and  $\beta$ -polysaccharides undergo much greater changes, such as complete hydrolysis, marked keturonic acid formation, and greater rotational changes. Furanosides are more reactive than pyranosides. While the differences noted do not parallel the rates of hydrolysis, yet the convergence of hydrolytic rates of  $\alpha$ - and  $\beta$ -glucosides at higher temperatures finds its counterpart in greater keturonic acid formation. This accounts for the better differentiation by naphthoresorcinol tests and optical rotations at lower temperatures.

**The Effect of Inorganic Salts on the Detoxication of Benzoic Acid in Rats.** BY MICHAEL SHEPPECK AND WENDELL H. GRIFFITH.

*From the Laboratory of Biological Chemistry, St. Louis University School of Medicine, St. Louis*

Earlier experiments demonstrated that certain inorganic salts protected young rats against the toxic effects of sodium benzoate if the benzoate and the salts were mixed with the ration. It was evident, therefore, that the detoxicating mechanism was subject to other factors in addition to the availability of glycine.

In the present study the rôle of the kidney, as one of the possible factors, has been examined. The rate of elimination of free benzoic acid, of hippuric acid, and of total combined benzoic acid in the urine of rats has been determined after the parenteral administration of sodium benzoate. The rate of elimination of hippuric acid was increased by the addition of glycine but was not affected by variations in the volume or tonicity of the benzoate solution or by the addition of those inorganic salts which had been shown to be effective in the earlier experiments. Furthermore, there was no relation between the excretion of hippuric acid and the volume of urine. The results revealed an interesting difference between the oral and parenteral administration of benzoate and indicated that the unusual effect of inorganic salts on the toxicity of benzoate was probably not related to their diuretic action on the kidney, since marked variations in the urine volume were without effect on the excretion of free and combined benzoic acid. These experiments do not exclude the possibility that certain salts decrease the toxicity of benzoate by a mechanism which is not directly related to the synthesis of combined benzoic acid.

**Determination of Vitamin A in Butter Fat.** BY LEO A. SHINN AND C. A. CARY. *From the Division of Research Laboratories, Bureau of Dairy Industry, United States Department of Agriculture*

The spectral absorption of the non-saponifiable fraction of butter fat is not the same as that for vitamin A, even after allowing for the absorption of the carotene and xanthophyll. It has been assumed that the other materials present in the butter fat do not

affect the absorption at  $\lambda$  325 to 328  $m\mu$ , and that this, when corrected for the carotene and xanthophyll, is a measure of the vitamin A. We were unable to find satisfactory evidence in the literature for this view.

Removal of the sterols that are insoluble in  $\text{CH}_3\text{OH}$  or  $\text{C}_2\text{H}_5\text{OH}$  at about  $-70^\circ$ , or their precipitation with digitonin, does not materially improve the agreement between the absorption of the remaining fraction of the non-saponifiable portion of butter fat and that of vitamin A. However, by shaking a ligroin solution of butter fat with  $\text{CH}_3\text{OH}$ , as is done in the estimation of sterols in fats, or by shaking the ligroin solution of butter fat with  $\text{MgO}$ , we have been able to remove material from the butter fat that does improve this agreement, especially between  $\lambda$  310  $m\mu$  and  $\lambda$  350  $m\mu$ , and have obtained evidence that this material does not absorb at  $\lambda$  325 to 328  $m\mu$ . Work on this fraction is continuing.

In addition, we have found that vitamin A, when added to butter fat, is recovered quantitatively in the ordinary spectrophotometric method, and that this recovery is not affected by the presence of cholesterol, ergosterol, or calciferol.

**The Metabolism of Pyrimidines in the Growing Dog.** By BERNARD E. SILVER AND LEOPOLD R. CERECEDO. *From the Department of Chemistry, Fordham University, New York*

The chemical relationships between purines and pyrimidines are well known. In order to ascertain whether pyrimidines serve as precursors of purines in the animal body, uracil, thymine, and cytosine have been administered to puppies, kept on a diet of known composition. We reasoned that if synthesis of purine bodies took place, such synthesis might be more evident in the growing than in the adult animal. Our observations show that these pyrimidines, when fed in small amounts (1.5 to 2.0 gm.), do not influence the output of urinary allantoin.

Recent workers claim to have observed a rise in the creatinine output of dogs after the administration of yeast nucleic acid. Determinations of urinary creatine and creatinine after the ingestion of the above pyrimidines failed to show an effect of these substances on the creatine-creatinine output.



**Evidence of the Synthesis of Essential Unsaturated Fatty Acids by the Rat.** BY ROBERT GORDON SINCLAIR. *From the Department of Biochemistry and Pharmacology, The University of Rochester School of Medicine and Dentistry, Rochester, New York*

Although the growth of rats on a diet devoid of highly unsaturated acids is distinctly subnormal, it is nevertheless very considerable. The source of the essential unsaturated acids which must have been available has remained unsettled. The following findings offer a solution of the problem.

Young rats were fed on a diet of casein, salt mixture,<sup>30</sup> yeast, and elaidin (74 per cent of the calories), supplemented with cod liver oil concentrate. After 3 to 4 weeks, when the weight was 80 to 90 gm., growth ceased. The weight remained constant for some weeks and then a gradual decline set in, terminating in death at the age of about 4 months. When growth had been arrested for a few weeks, some of the rats were fed a diet in which sucrose replaced the elaidin. Immediately growth was resumed at the rate of about 20 gm. a week. Such rats eventually reached the same weight as that of rats fed from weaning age on the same "fat-free" diet.

Replacement of about 5 per cent of the elaidin by a 1:1 mixture of cod liver and corn oils resulted in excellent growth, showing that elaidin in large amounts is not toxic.

It is thought that the high intake of elaidin—and presumably of other fats devoid of essential unsaturated acids—almost completely abolishes the limited synthesis of these highly unsaturated acids from carbohydrate and thus prevents growth. Other possible but less probable causes of the poor growth are being studied.

**Further Evidence of the Existence of Metabolic and Non-Metabolic Phospholipids.** BY ROBERT GORDON SINCLAIR. *From the Department of Biochemistry and Pharmacology, The University of Rochester School of Medicine and Dentistry, Rochester, New York*

Elaidic acid has been used for the study of the rate of phospholipid turnover in various tissues of the cat, especially with reference to the rôle of the blood phospholipids in fatty acid transport.

<sup>30</sup> McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).

Within a few hours after feeding elaidin, (a) the phospholipids of the intestinal mucosa contain large amounts of elaidic acid, thus confirming the rapid and extensive taking up of ingested fatty acids previously deduced from the change in the degree of unsaturation; (b) as high as 28 per cent of the fatty acids in the phospholipids of the blood plasma consists of elaidic acid, thus confirming in a very direct way the long assumed transport function of plasma phospholipid; (c) no elaidic acid was detected in the phospholipids of the red blood cells, indicating that, in the cat at any rate, they do not participate in fatty acid transport; (d) the phospholipids of the liver contain considerable amounts of elaidic acid and therefore undergo a rapid turnover; (e) the kidney phospholipids contain no elaidic acid, indicating a slow rate of turnover and thus a non-metabolic function.

**The Action of Iodoacetate and Iodoacetamide on Certain Sulfhydryl Groups, on Urease, and on Fermentation.** By C. V. SMYTHE. *From the Laboratories of The Rockefeller Institute for Medical Research, New York*

Although crystalline urease is remarkably resistant to the action of iodoacetate,<sup>31</sup> I have found that it is readily inactivated by iodoacetamide. In seeking an explanation of this finding I have measured the rate of reaction of these two iodo compounds with thioglucose, thiosalicylic acid, cysteine, glutathione, and thioglycol. In each case the iodoacetamide reacts more rapidly. The order of the rates for the various sulfhydryl compounds is the order given, the thioglucose reacting about 30 times as rapidly as the thioglycol. Neither of the two iodo compounds reacts at a measurable rate with ethyl mercaptan under physiological conditions. The rate of the reaction of both these iodo compounds with urease has been measured. From these curves, which presumably give the number of sulfhydryl groups destroyed, and the measurements of the activity of the enzyme, it is possible to calculate the number of sulfhydryl groups that must be destroyed in order to inactivate the enzyme and, from this, a minimum value for the molecular weight of the enzyme.

A comparison of these two iodo compounds as inhibitors of

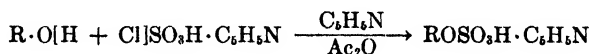
<sup>31</sup> Hellerman, L., Perkins, M. E., and Clark, W. M., *Proc. Nat. Acad. Sc.*, **19**, 855 (1933).

alcoholic fermentation by yeast cells and by cell-free extracts shows the iodoacetate to be a considerably more rapid inhibitor than iodoacetamide. Thus the activity of the two compounds as inhibitors of fermentation in yeast preparations is just the reverse of their activity on urease and on certain sulfhydryl groups.

**The Quantitative Isolation of Small Quantities of "Free Cholesterol" As the Pyridine Cholesteryl Sulfate.** BY ALBERT E. SOBEL, I. J. DREKTER, AND SAMUEL NATELSON. *From the Pediatric Research Laboratory, The Jewish Hospital of Brooklyn, and the Achelis Laboratory, Lenox Hill Hospital, New York*

The hitherto available methods for the separation of "free cholesterol" from its esters in biological materials depend upon the isolation of the cholesterol digitonide. However, the chemical reaction between cholesterol and digitonin is still obscure. Digitonin of uniform composition is not easily obtained, so that various samples of this reagent may give different results. Moreover, digitonin affects the Liebermann-Burchard colorimetric reaction.

The disadvantages of the digitonin method were obviated by developing the conditions necessary for the isolation of small quantities (0.04 mg.) of cholesterol as its pyridine sulfate derivative. The reaction between cholesterol and pyridine chlorosulfonate is illustrated in the equation



(R = sterol residue.)

The above reaction goes to completion in a benzene medium at 45–47° in the presence of a 1:1 mixture of pyridine and acetic anhydride. The pyridine cholesteryl sulfate is isolated by the addition of petroleum ether (35–60°), in which the above salt is insoluble. The excess of pyridine chlorosulfonate is also precipitated. This, however, does not matter, since the precipitate neither interferes with nor affects the intensity or shade of the color produced in the Liebermann-Burchard reaction, which is used in the final estimation. The petroleum ether washings contain all the esterified cholesterol. This may be determined subsequent to saponification.

This procedure was applied to the lipid extract of blood serum.



**Paradoxical Ketosis.** BY MICHAEL SOMOGYI. *From the Laboratory of the Jewish Hospital of St. Louis, St. Louis*

The observation was made that ketosis may occur in human diabetics at times when, aided by the administration of insulin, they utilize substantial quantities of carbohydrates, such as are far in excess of the antiketogenic requirement. Thus, in cases where the difference between ingested carbohydrate and glucose spilled in the urine amounted to 200 and well over 200 gm. in 24 hours, 1 to over 5 gm. of total acetone bodies were found in the urine corresponding to the same period.

In several instances the occurrence of severe ketosis was observed immediately following hypoglycemic shock in insulin-treated diabetic patients, who for some time prior to the insulin shock were well under control on diets containing 120 to 250 gm. of carbohydrates with commensurate doses of insulin.

**The Relationship between Total and Free Cholesterol in Human Blood Serum.** BY WARREN M. SPERRY. *From the Chemical Laboratory, Babies Hospital, and the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

The concentration of total and free cholesterol was determined by the method of Schoenheimer and Sperry<sup>22</sup> in 126 samples of blood serum from 91 healthy, adult, human subjects. The minimum percentage of free in total cholesterol was 24.3 per cent, the maximum was 30.1 per cent, and the average was 26.9 per cent, with a standard deviation of 1.4 per cent. The result leads to the conclusion that the percentage is far more constant than has been recognized by previous investigators, most of whom have reported much larger variations and a higher average. Support for this conclusion was obtained in the finding that a large proportion of samples taken *post mortem* from humans who had died suddenly gave values in the same narrow range. Similar results were obtained in healthy children and in four-fifths of a large series of determinations on diseased children. With few exceptions values above 31 per cent were found only in the presence of infection or liver disease.

<sup>22</sup> Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, **106**, 745 (1934).

The percentage of free in total cholesterol appears to be a physiological constant which may be of considerable value in the study of cholesterol metabolism and in pathology.

**Further Studies of Polarization in Tissue Models.** BY MONA SPIEGEL-ADOLF. *From the Department of Colloid Chemistry, D. J. McCarthy Foundation, Temple University School of Medicine, Philadelphia*

To study biological problems on tissue models from a physico-chemical and a chemical point of view, the construction of polarizable membranes with low resistance and of membranes in which the chemically reactive parts are constituents of the animal body was tried.

In continuing former studies it could be shown that besides lipids (lecithin, cephalin) other substances of very different chemical character such as olive oil, mastix, benzoe gum, act in the same way. When they were added to collodion, the membrane made of the mixture showed polarization, which a pure collodion membrane made in the same way did not.

By preventing the shrinkage of drying collodion membranes through pressure, a variation of polarization and resistance within a wide range, including biological conditions, has been achieved. By this treatment a collodion membrane lacking polarization can without chemical changes be endowed with this property. Comparison of unpolarizable and polarizable membranes showed that acids, alkalies, salts with bi- and trivalent ions produce effects similar to those observed in living tissue in the second group only.

Furthermore, the relations between polarization and permeability were investigated, the results justifying the use of polarization as a measure for permeability.

Endeavors to build up solid protein or protein-lipid membranes showing polarization failed. Yet by adsorption of protein and lipids to the sintered glass plate of a Jena glass Gooch crucible a membrane was produced that did possess polarizing effects. These could be demonstrated by measuring the electrical conductivity at high and low frequencies and by directly determining the membrane potential.

**The Effect of the Quantity of Vitamin D Intake upon Calcium Retention in Infancy.** BY GENEVIEVE STEARNS AND P. C. JEANS. *From the Department of Pediatrics, College of Medicine, State University of Iowa, Iowa City*

Studies of calcium retention of two groups of infants, one given approximately 340 U.S.P. units of vitamin D daily, the other given milk containing 135 U.S.P. units of vitamin D per quart, are compared with studies from the literature concerning calcium retention of artificially fed infants receiving no vitamin D. Cod liver oil and irradiated milk were the sources of vitamin D.

As the intake of vitamin D increased, the average retention of calcium increased for any given per kilo intake, and the range of observed retentions narrowed. The effect of increased vitamin D was more marked at the lower levels of calcium intake, but the average retention of calcium with 340 units of vitamin D was greater at all levels studied than the average retention with 135 unit milk. The chief result of increased vitamin D intake seems to be in increasing the average level of retention of each infant to that of the best retentions obtained without added vitamin D.

Abrupt changes in intake level may or may not be followed by abrupt changes in retention. Two infants whose intakes of vitamin D were increased abruptly showed practically no change in retention for 2 months after the increase in vitamin D intake. With other infants, similar abrupt changes in vitamin D intake were followed by exaggerated changes in retention. Similar variations have been observed during recovery from minor illnesses. Because of these findings, short term experiments upon infants without knowledge of previous dietary or health conditions are considered of doubtful value.

**The Comparative Rate of Absorption of Various Fats.** BY HARRY STEENBOCK, MARGARET HOUSE IRWIN, AND JANET WEBER. *From the Department of Agricultural Chemistry, University of Wisconsin, Madison*

A comparison of the rate of absorption of a number of fats was made. Rats were fasted 48 hours, fed 1.5 cc. of melted fat by stomach tube, and after absorption periods of 2, 4, 6, 8, and 12 hours the amount of fat absorbed was determined by analyzing the intestinal residues. The percentage absorption of the various

fats after a 4 hour absorption period was as follows: two partially hydrogenated vegetable oils,  $52.8 \pm 2.4$  and  $53.8 \pm 1.6$  per cent, respectively; lard,  $57.0 \pm 1.5$  per cent; corn oil,  $58.3 \pm 0.9$  per cent; butter oil,  $71.0 \pm 1.2$  per cent; halibut liver oil,  $70.2 \pm 2.0$  per cent; and cod liver oil,  $67.7 \pm 1.9$  per cent. It was found that partially hydrogenated vegetable oils, as sold commercially for home and bakers' use, were absorbed as rapidly as lard or corn oil, and that butter oil, halibut liver oil, and cod liver oil were absorbed uniformly at a more rapid rate than lard, corn oil, or the partially hydrogenated fats.

**Sexual Differentiation in the Storage of Iron by the Rat.** BY HARRY STEENBOCK, JOSEPH SEMB, AND EVELYN C. VAN DONK.  
*From the Department of Agricultural Chemistry, University of Wisconsin, Madison*

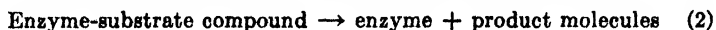
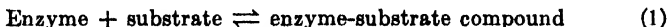
Gustav Bunge in 1889 revealed the significant fact that of the various elements needed by the growing mammal iron stands out as one which is transmitted to the young through the placenta rather than through the mammary gland. We have submitted the storage of iron to further analysis. The rat was used as the experimental animal. From our experiments it is evident that the amount of iron in the young at birth stands in direct relation to the amount stored by the mother. Litters sometimes varied considerably in their stores, but normal litter mates differed but little. However, as the young attained sexual maturity, the females accumulated larger stores than the males. During pregnancy these stores were rapidly depleted as the amount of iron in the fetuses increased. After parturition the depleted stores were rapidly built up to their former level.

It appears that the storage of iron in the female is subject to the influence of ovarian secretions. Not only did the differentiation in stores make its appearance only with the attainment of sexual maturity, but castration of the females resulted in decreased storage. Injection of follicular hormone (progynon, Schering) into sexually immature females led to a greater storage than occurred in litter mate controls.

**On the Mechanism of Enzyme Action.** BY KURT G. STERN.  
*From the Department of Physiological Chemistry, Yale University, New Haven*



Direct spectroscopic observation of the action of catalase on monoethyl hydrogen peroxide<sup>33</sup> permits an experimental analysis of the two main steps of the enzyme reaction:



Reaction (1) is amenable to spectroscopic study. The over-all process ((1) + (2)) is followed by volumetric determination of the peroxide.

The rate of reaction (1) is great compared with that of the total process but still measurable (of the order of seconds). The limiting factor is the rate of reaction (2) (of the order of minutes). A ratio of about  $10^5$  substrate molecules to 1 enzyme molecule is required to shift the equilibrium in reaction (1) to the right. This renders the speed of the over-all process maximal. The average life of a single enzyme-substrate particle may well be of the order of  $10^{-7}$  second as calculated by Haldane.<sup>34</sup> Reaction (1) has a small temperature coefficient ( $Q_{10} = 1.26$ ,  $0-20^\circ$ ) compared with that of reaction (2) ( $Q_{10} = 2.2$ ,  $0-20^\circ$ ), and therefore probably a smaller energy of activation. The apparent critical increment for reaction (1) + (2) is  $E = 13,000$  calories between  $0-20^\circ$ . The rate of formation of the enzyme-substrate compound is independent of pH between 4.1 and 8.8.

The linkage between the porphyrin-bound trivalent iron of the enzyme and the peroxide appears to be of a coordinative character. Preceding the appearance of the spectrum of the intermediate the enzyme seems to undergo some molecular rearrangement. The product molecules of reaction (2) need not necessarily be the final products (*e.g.* acetaldehyde) but may be radicals initiating a reaction chain which proceeds without the further participation of the enzyme.

### Chemical Studies of the Pernicious Anemia Principle in Liver.

BY YELLAPRAGADA SUBBAROW AND B. M. JACOBSON. *From the Biochemical Laboratory, Harvard Medical School, and the Medical Clinic, Massachusetts General Hospital, Boston*

<sup>33</sup> Stern, K. G., *Nature*, **136**, 335 (1935).

<sup>34</sup> Haldane, J. B. S., *Proc. Roy. Soc. London, Series B*, **105**, 559 (1931).

A study of the chemical fractionation of extracts of liver and their effect on pernicious anemia has been continued. The results on eight patients indicate that at least three chemically distinct fractions are necessary for an optimum clinical response.

One fraction has been identified as *l*-tyrosine. A second fraction has been crystallized and shown to be a purine of complex nature. The third fraction, free from tyrosine and the purine, contains a substance which reacts as if it were a polypeptide. The amount of this fraction derived from 100 gm. of fresh liver contains approximately 3 mg. of nitrogen. It yields crystalline salts with rhodanilic acid and Reinecke's salt. The rhodanilate is precipitated by phosphotungstic, picric, rufanic, and tannic acids as well as by ammonium sulfate. It gives positive biuret, diazo, and glyoxalic reactions; but is negative with Millon's reagent. The Reineckate is precipitated by phosphotungstic and rufanic acids. It gives a ninhydrin and a weak diazo reaction.

**Further Studies on Muscular Dystrophies.** BY M. X. SULLIVAN.

*From the Chemo-Medical Research Institute, Georgetown University, Washington*

Work on the possible relation of simple guanidine derivatives to muscular dystrophies has been continued. To date urinary examinations have been made on six cases of progressive muscular dystrophy, four of pseudohypertrophic muscular dystrophy, and three of myasthenia gravis. By oxidation with  $\text{Ag}_2\text{O}$  free guanidine was obtained from eight of the ten cases of muscular dystrophy, while no guanidine was found in myasthenia gravis. Normal urines have never delivered guanidine under the same procedure. With the giving of glycoral pills over a long period to four cases of muscular dystrophy the simple guanidine derivatives disappeared from the urine, the appetite increased, and gain in weight resulted, but judgment is suspended as to whether the dystrophy is checked or improved.

**Method for Measurement of Serum Volume.** BY F. WILLIAM SUNDERMAN AND J. HAROLD AUSTIN. *From the John Herr Musser Department of Research Medicine, University of Pennsylvania, Philadelphia*

An apparatus for the intravenous injection of vital red and the

method of colorimetry employed in the measurement of this dye in serum are demonstrated. The method permits precise measurement of the dye introduced into the vein, allows time for homogeneous mixing, takes into account the rate of disappearance of the dye from the serum, and permits of an accurate measurement of the concentration of the dye present in the serum, regardless of species. Studies of the concentration of hemoglobin present in serum which would interfere with the measurement of vital red have been made.

**Spectrophotometric Studies of the Color of Solutions. I. Selection and Application of Light Filters in the Analysis of Blood Sugar by the Benedict Method and of Cholesterol by the Liebermann-Burchard Reaction.** BY F. WILLIAM SUNDERMAN AND JOSEPH RAZEK. *From the John Herr Musser Department of Research Medicine, University of Pennsylvania, Philadelphia*

The development of color in these solutions was studied by means of a photoelectric spectrophotometer which permitted the spectral transmission at each wave-length to be obtained in about 10 seconds. The first curve was obtained at 2 minutes after preparation of a given solution and subsequent curves were obtained at intervals up to 1 hour. From these studies it was possible to isolate the wave-length zone at which transmission was most constant over a considerable period of time and to select appropriate combinations of filters for the direct colorimetry of the solutions studied.

**Toxic Effect of Cod Liver Oil in the Ration of the Rabbit and the Calf.** BY WILLIAM A. TURNER, EDWARD B. MEIGS, AND H. T. CONVERSE. *From the Division of Dairy Research Laboratories, Bureau of Dairy Industry, United States Department of Agriculture*

When rabbits are taken at 60 days of age from a stock ration and given 20 gm. daily of a good grain mixture with low grade roughage (U. S. No. 3 alfalfa or timothy hay) *ad libitum*, they have about an even chance of survival to 180 days of age. If, however, cod liver oil, as 5 per cent of the grain component (about 0.7 gm. per kilo of body weight daily), is added to the ration, death will almost certainly occur between 90 and 150 days of age.

Stiffness of limbs and emaciation precede death. Histological examination shows degeneration of the muscle tissue.

If the roughage consists of good quality alfalfa hay, the animals survive and growth and reproduction appear only partially impaired by the cod liver oil feeding.

Young calves, receiving a good grain mixture with skim milk (from cows not on pasture) and U. S. No. 3 timothy hay, do not survive. When cod liver oil is supplied as not more than 0.7 gm. per kilo of body weight daily, the calves survive and grow normally. Administration of 2.0 gm. or more of cod liver oil per kilo of body weight daily causes death.

**The Synthesis and Depressor Effect of *d*-Carnosine, the Enantiomorph of the Naturally Occurring Form.** BY VINCENT DU VIGNEAUD AND MADISON HUNT. *From the Department of Biochemistry, School of Medicine, George Washington University, Washington*

Many instances of the relationship of physiological activity to spatial configuration, such as in the case of *d*- and *l*-adrenalin and of certain amino acids, are a matter of record. The present study was undertaken to find out whether such a relationship existed in the case of carnosine.

*d*-Carnosine was synthesized by the method utilized by Sifferd and du Vigneaud for the naturally occurring isomer. The azide of carbobenzoxy- $\beta$ -alanine was condensed with *d*-histidine methyl ester and the resulting *d*-carbobenzoxycarnosine methyl ester was saponified and reduced. The *d*-carnosine was isolated both as the free crystalline peptide and as the hydrochloride.

The *d*-histidine used in the above synthesis was obtained by the resolution of *dl*-histidine with tartaric acid. *l*-Histidine was acetylated and racemized by the method of du Vigneaud and Myer by treating it in aqueous solution with acetic anhydride in the presence of sodium acetate. The acetyl-*dl*-histidine was hydrolyzed with HCl and the hydrochloride converted to free *dl*-histidine.

Preliminary tests in comparison with *l*-carnosine indicated that *d*-carnosine possessed little if any depressor action on the blood pressure of the cat, thus demonstrating that the depressor effect of carnosine is dependent upon spatial configuration. More

complete studies of the physiological behavior of this unnatural isomer are under way and will be reported.

**Studies on the Metabolism of Sulfur in Cats.** BY ROBERT W. VIRTUE. *From the Department of Biochemistry, Louisiana State University Medical Center, New Orleans*

The bile and urine of well fed and fasting cats were analyzed 24 hours after subcutaneous injection of monobromobenzene in doses of 1.5 gm. per kilo. The quantity of alcohol-soluble sulfur found per gm. of bile was less than that found in bile taken from control animals. Urinary sulfur partitions gave values in accord with the supposition that part of the injected monobromobenzene had been conjugated with cysteine and excreted as a mercapturic acid. *p*-Bromophenylmercapturic acid was isolated from this cat urine and identified as such. In the fasting animals the cysteine for this conjugation must have been of endogenous origin. This cysteine may have arisen from a sulfur-containing moiety which normally would have formed a portion of the alcohol-soluble sulfur fraction of the bile.

Urine from well fed and from fasted cats which had been given methionine subcutaneously showed the presence of disulfide groups, but gave negative Sullivan tests for cystine.

**The Effect of Insulin on Liver Glycogenase.** BY G. K. VOLLMAR AND ALFRED E. KOEHLER. *From the Santa Barbara Cottage Hospital and the Sansum Clinic, Santa Barbara*

Glycogenase was extracted from rat livers by means of 25 per cent glycerol in saline buffered at pH 7.0. The supernatant fluid of the centrifuged extract was precipitated with 10 volumes of alcohol and the precipitate extracted with saline. The glycogenase was estimated by the amount of glucose liberated from glycogen upon incubation.

The glycogenase activity of the supernatant glycerol extract increased with incubation up to a period of 20 to 30 hours. This phenomenon of increase was absent or at a minimum (7 per cent) in the livers of a group of thirty-seven fasting rats, increased (56 per cent) after intraperitoneal injection of glucose before killing in eighteen rats, and was most marked (65 per cent) in a group of twenty-seven fed rats.

The possibility that glycogenase increase on incubation was due to autolytic liberation of the enzyme could not be excluded, although the fact that glucose injection altered the rate of increase raises the question of change in the enzyme itself, possibly inversion from the glycogenic to the glycogenolytic form. That lipase did not increase with incubation is another argument against the theory of autolytic liberation of glycogenase.

Insulin in amounts of 8 units per rat increased the liver glycogenase in a group of eighteen fasting rats over a similar control group by 39 per cent. " In a group of thirteen non-fasting rats the insulin increased the glycogenase by 35 per cent. The increase in liver glycogenase is consistent with decreased liver glycogen after insulin in the non-diabetic animal.

**Studies on the Metabolism of Guanidoacetic Acid.** BY C. J. WEBER. *From the Department of Internal Medicine, University of Kansas School of Medicine, Kansas City*

The presence of guanidoacetic acid in the urine places this substance in the rôle of a possible normal metabolite of the human and animal body. Analysis of 24 hour urine samples of human subjects gives an average excretion for males of 39.5 mg. and for females of 75.1 mg. Analysis of blood and tissues indicates that only traces of guanidoacetic acid are present. The body has a definite but limited tolerance for administered guanidoacetic acid and the excess is readily excreted in the urine.

**The Composition of Growth Induced by Vitamin B (B<sub>1</sub>).** BY DOROTHY V. WHIPPLE AND CHARLES F. CHURCH. *From the Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia*

In paired feeding experiments animals on fat-free diets receiving vitamin B (B<sub>1</sub>) were able to gain more weight than their mates on the same fat-free diet but without the vitamin. The average body compositions of the animals receiving vitamin B and their mates on isocaloric intakes but not receiving the vitamin were (in per cent) as follows: with vitamin B, water 64.9, fat 9.4, protein 18.5; without vitamin B, water 67.8, fat 3.3, protein 21.2. The animals receiving vitamin B (B<sub>1</sub>) gained 13.5 gm. more in weight than the animals not receiving the vitamin. Fat accounted for 6.5 gm. and water for 6.2 gm. of this total.

These figures suggest the possibility that vitamin B ( $B_1$ ) plays a rôle in the synthesis of fat in the animal body.

**The Determination of Carotene in Fresh Plant Materials.** BY HERBERT G. WISEMAN AND EDWARD A. KANE. *From the Division of Research Laboratories, Bureau of Dairy Industry, United States Department of Agriculture*

Various methods of estimating carotene in freshly cut plant materials have been investigated, and a method has been developed with the view of reducing to a minimum the destruction of the carotene which occurs readily in such materials.

Although carotene is in part destroyed when dry plant materials are ground to a powder in a ball mill, we have found that this destruction is prevented if the material is suspended in alcohol. In determining the carotene in fresh plant material, absolute alcohol is taken into the field and cooled to about  $-70^\circ$  by immersing dry ice in it; with a large excess of dry ice still in the alcohol, the freshly cut material is immediately immersed in it, and the frozen tissues broken up with a pestle. A large quantity of dry ice is added to the mixture, which is then allowed to stand overnight in a refrigerator at  $0-5^\circ$ . The alcohol is then filtered off and the solid residue, suspended in fresh absolute alcohol, is ground to a fine powder in a ball mill. The alcohol is again filtered off and the residue, washed with absolute alcohol and then with ligroin ( $30-60^\circ$ ), is practically free of carotene. The carotene from the combined filtrates and washings is taken up into the ligroin and determined as usual.

This method has been compared with other methods and appears most satisfactory in quantity of carotene found, ease of separation of the pigments, and the light absorption curve of the final carotene fraction.







